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OBSERVATIONS ON THE COURSE OF INFECTION WITH CERTAIN NATURAL BACTERIAL PATHOGENS OF THE RAT IN RATS ON PROTEIN-DEFICIENT DIETS.

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HIMSWORTH and Glynn in 1944 reported that rats on diets grossly deficient in protein developed acute necrosis of the liver and that they could be protected by addition of sulphur-containing amino-acids (Himsworth and Glynn, 1944*a*, *b* and *c*; Glynn and Himsworth, 1944). MacCallum and Miles (1946), using a diet suggested by Himsworth, which was supposed to contain the minimum amount of casein necessary to protect rats' livers from acute necrosis for at least 60 days, attempted to pass infective hepatitis to albino rats.

In both the work of MacCallum and Miles and that of Himsworth and Glynn (Himsworth, personal communication) difficulty was experienced from the high rate of bacterial infection in rats on protein-deficient diets, and numerous experiments had to be repeated because of such casualties. In the former work rats were obtained from two different sources, and those from the one source were commonly infected with *Salmonella typhyi-murium*, while those from the other succumbed to a diphtheroid which is named *Corynebacterium murium* or *Coryne bacterium pseudotuberculosis murium* (Wilson and Miles, 1946). The latter organism was originally described by Klein (1903), and was said to have a particularly high pathogenicity for rats on inadequate diets (Mitchell, 1912).

Neither of these organisms attacks the liver predominantly, although both are likely to produce hepatic lesions, and we therefore considered the possibility that the type of diet which we were using was not just one which would make the liver more susceptible to infection, but might be associated with a more general reduction in resistance to infection.

Experiments were therefore designed to show whether the protein-deficient diets which we were using would impair the ability of rats to form agglutinins against bacteria, and whether such diets would impair the ability of rats to form complement. It was planned to test the same animals for resistance to infection in an attempt to correlate complement or antibody titres with resistance to infection.

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MATERIALS AND METHODS.

Rats.

The albino rats used in these experiments were bought from one breeder. They were received when weighing 70–80 g. and were used immediately.

Bacterial strains.

Salm. typhi-murium.—The strain used was obtained from a rat dying from this infection. The organism was subcultured daily, normally on agar slopes, but every seventh day it was plated out on agar and 10 typical smooth colonies were picked for propagation. Usually the plate cultures showed only smooth colonies, but on one occasion 10 per cent were found to be rough.

C. murium.—The strain used was obtained in pure culture from the lung lesions of a rat dying of pseudo-tuberculosis due to this organism. The cellular and colonial morphology and the biochemical reactions were the same as those given for *C. murium* by Wilson and Miles (1946), but the organism had a very low pathogenicity for mice. Large doses produced a local abscess after subcutaneous inoculation, but no general disease developed and no lesions were found after intraperitoneal inoculation.

Vaccine.

The suspension of *Salm. typhi-murium* used for immunization of rats was taken from a 15-hour agar slope culture, killed by heating to 56° for 30 minutes and standardized to 10^9 organisms per ml. Its sterility was verified.

Diets.

Stock diet.—The stock diet for the rats was of commercial cubes which had a slightly variable analysis, but contained approximately 16 per cent of protein. Water was added. Animals fed on this diet gained weight satisfactorily.

Protein-deficient diets.—The basic protein-deficient diet was that which MacCallum and Miles had used on the advice of Himsworth (Himsworth and Glynn, 1944*b*). This diet contained 4 per cent casein and 16 per cent yeast, made up with corn starch and arachis oil with vitamin and salt supplements.

Usually animals were kept six to a cage, and they were given sufficient food to allow each animal 8 g. a day. It was not possible to avoid coprophagy. All animals were allowed water *ad lib.* The animals ate the diet readily.

This diet alone does not cause liver lesions to appear in albino rats in a shorter time than 60 days, and then only a very occasional rat may show small areas of liver necrosis. After 122 days one rat developed massive acute necrosis of the liver apparently due to this diet alone.

When the amount of casein in the diet was varied the increases were balanced by an equivalent decrease in the amount of corn starch. The animals were used after varying periods on these diets, and controls on stock diet were from the same batch of rats.

Blood samples.

During the course of the experiments blood samples were taken from rats by cardiac puncture, the animals being anaesthetized with ether. Rats which survived to the end of the experiments were bled out by cutting the brachial artery and the blood was collected with a pasteur pipette.

Complement titrations.

Fresh serum was diluted to 1 : 15 with normal saline and dilutions were made from this by pipetting quantities of diluted serum from 0·12 ml., diminishing in each tube by 0·01 ml. to 0·01 ml., and adding normal saline to make up a total volume of 0·2 ml. (Ecker and Pillemer, 1942). These dilutions were made using 0·2 ml. "Kahn" pipettes graduated to 0·001 ml., and 0·4 ml. of 3 per cent sheep's R.B.C. sensitized with 5 minimal haemolytic doses (MHD) of haemolysin was added to each tube. Serum controls were added containing 0·12, 0·06 and 0·03 ml. of serum. Cell controls were included.

The tubes were incubated in a water-bath at 37° for 45 minutes and remaining R.B.C. were deposited by centrifugation. The end-point was taken as the last tube showing definite haemolysis. The complement titre has been expressed as the number of MHD contained in 1 ml. of undiluted fresh serum. All complement titrations were carried out on the day on which the animals were bled.

Agglutinations.

Salm. typhi-murium agglutination tests were set up against standard H and O suspensions of the organism obtained from the Public Health Laboratory Service.

For agglutinations with *C. murium*, live suspensions were made in normal saline up to the same opacity as the *Salmonella* suspensions. These suspensions were found to be stable.

Sera were inactivated by heating to 56° for 30 minutes and serial doubling dilutions were made from an initial dilution of 1 : 10. Agglutinations were carried out using 0·3 ml. volumes of the reagents in Dreyer's tubes incubated in a water-bath at 50°. The results of the *Salmonella* H agglutinations were read after four hours, and those of the O agglutinations and the *C. murium* agglutinations after 18 hours. The end-point was taken as the last tube in which agglutination was clearly visible with the hand lens, and expressed as the dilution of serum in that tube before the addition of the bacterial suspension.

Methods of infection.

All inoculations were made using saline suspensions from 18-hour cultures on solid media. The suspensions were standardized using Brown's opacity tubes, and it was assumed that the opacity of the suspensions of *C. murium* bore the same relation to the number of organisms present as that of *C. diphtheriae*, since the cellular morphology of the 18-hour culture was similar to that of a *gravis* strain of *C. diphtheriae.*

Intraperitoneal inoculations were made in a volume of 1 ml., the concentration of the suspension being varied, and intranasal inoculations were made under ether anaesthesia using a volume of 0·2 ml.

EXPERIMENTAL.

Complement Titres in Rats on Stock Diet and 4 per cent Casein Diet.

Experiment 1.

Three rats which had been on 4 per cent casein diet and six which had been on stock diet for 21 days were bled and the complement titres examined. One

of the normal animals had less than 125 minimal haemolytic units of complement per ml., but if this animal is assumed to have 100 units per ml., then the mean titre of the normals would be 275 units per ml. and that of the diet animals 280 (Table I).

TABLE I.—*Titre of Complement in Rat Sera from Rats 21 Days on a Diet containing 4 per cent Casein and Controls.*

	Animals on stock diet.	Animals on 4 per cent casein diet.
Titre of complement in individual rats	<125	250
	375	215
	375	375
	250	
	250	
	300	
Mean	275*	280

* To obtain this figure it was assumed that the serum of rat 1 contained 100 minimal haemolytic units per ml.

TABLE II.—*Titre of Complement in Rat Sera at the Beginning of the Experiment and after 28 Days in Seven Rats on 4 per cent Casein Diet and Seven Rats on Stock Diet.*

	Animals on stock diet.		Animals on 4 per cent casein diet.	
	Day 1.	Day 28.	Day 1.	Day 28.
Titre of complement in individual rats	250	125	300	300
	215	250	375	300
	300	375	125	300
	300	300	300	215
	375	250	300	250
	140	300	190	215
	250	140	215	215
Mean	261	249	256	256

In both tables the titre is expressed as the number of haemolytic units present in 1 ml. of undiluted serum.

Experiment 2.

One batch of rats was divided into two groups of seven and all the animals were bled by cardiac puncture. One group was then placed on the 4 per cent casein diet and the other was kept on stock diet. After 28 days both groups were again bled. Both batches of sera were titrated for complement content on the day on which they were obtained. There were large variations in titre in individual rats in each group, but no trend was demonstrable. The mean titre before the experiment in the control group was 261 units per ml., and after the experiment it was 249. In the group on the inadequate diet the mean titre both before the experiment and on the 28th day was 256 (Table II).

These experiments may be criticized because rat serum often contains small amounts of natural haemolysin against sheep corpuscles, but when the sera are

titrated for minimal haemolysis, in nearly all cases the haemolysin is titrated out well before the complement and can have little effect on the result.

The results do not suggest that a protein-deficient diet adversely affects the complement content of rat sera. Possibly complement fractionation experiments might show that some fraction was present only in reduced quantity, but the clear-cut negative results obtained in these two experiments did not encourage any further work on these lines.

Production of Agglutinins for Salm. typhi-murium, *and Resistance to this Organism.*

Experiments were carried out to compare the antibody formation in rats on 4 per cent casein diet and controls on stock diet, and to compare their resistance when their immunity was challenged by intraperitoneal inoculation.

Experiment 3.

Four animals on the deficient diet and four controls on stock diet received a course of three weekly intraperitoneal injections of 10^8 killed *Salm. typhi-murium*, beginning at the time when they were put on the diet. One of the diet animals died during this period. All the remaining animals were bled 14 days after the last inoculation and the sera were titrated for somatic agglutinins. There was no difference between the titres in the two groups (Table III).

TABLE III.—*Effect of a 4 per cent Casein Diet on Titre of Somatic Agglutinins against* Salm. typhi-murium *in Rats which had Received Three Weekly Injections of Vaccine.*

	Animals on stock diet	Animals on 4 per cent. casein diet
Titre in individual rats	1:80	1:40
	0	1:40
	1:40	1:40
	1:40	—
Mean	1:40	1:40

TABLE IV.—*Effect of a 4 per cent Casein Diet on Titre of Flagellar and Somatic Agglutinins against* Salm. typhi-murium *in Rats which had Received Three Large Weekly Injections of Vaccine.*

	Animals on stock diet.		Animals on 4 per cent casein diet.	
	anti-" H."	anti-" O."	anti-" H."	anti-" O."
Titre in individual rats	1:12,800	1:800	1:6,400	1:400
	1:6,400	1:800	1:12,800	1:800
	1:12,800	1:1,600	1:6,400	1:200
	1:3,200	1:100	—	—
Mean	1:8,800	1:825	1:8,530	1:467

* In both tables the titre is expressed as the serum dilution giving standard agglutination.

Experiment 4.

Two groups of 6 rats were placed on 4 per cent casein diet and two groups of 4 rats from the same batch were kept as controls. One group of test and one of controls received the following course of *Salm. typhi-murium* vaccine : On day 1, 4×10^8 organisms i.p., on day 8, 8×10^8, and on day 15, 15×10^8. The remaining groups were not immunized. All rats were bled at the beginning of the experiment and fourteen days after the last inoculation. During this preliminary period one control from the non-immunized group and five protein-deficient animals died, and during the final bleeding another protein-deficient rat succumbed, but fortunately these deaths were equally divided between the groups.

On the day following the final bleeding the unimmunized animals received an inoculation of 10^9 live *Salm. typhi murium* i.p. and the immunized $2{\cdot}5 \times 10^9$ organisms.

TABLE V.—*Deaths from Infection with* Salm. typhi-murium *when Immunized and Unimmunized Animals on* 4 *per cent Casein Diet and Controls were Inoculated Intraperitoneally with Live Suspensions.*

Dose of organism.	Group of rats.	Time to death in hours.	Mean.
1000×10^6	Unimmunized 4 per cent casein diet	8, 24, 46	26
	Unimmunized controls	72, 96, 104	91
2500×10^6	*Immunized 4 per cent casein diet	72, 120, 120	104
	*Immunized controls	120, 168, s, s	—

s = Survived.

* These are the same animals whose agglutinin titres are recorded in Table IV.

The results are shown in Tables IV and V. None of the animals showed any agglutinins at a dilution of 1:10 against the organism before immunization. Although the mean agglutinin titres of the protein-deficient group are slightly lower than those of the control group, the individual variations are so great that such a small difference is without any significance. However, in both the immunized and the unimmunized groups, when the rats were inoculated with living organisms the animals on adequate diet proved consistently more resistant than those on the protein-deficient diet.

The groups are small and the experiment has not been repeated, but the result goes to confirm the clinical impression which we had obtained that rats on protein-deficient diets are more susceptible to *Salmonella* infection than normal rats. Further, it does not appear that this difference in susceptibility can be closely related to differences in the ability to produce agglutinins demonstrable *in vitro*.

Experiments on Resistance to C. murium.

Experiment 5.

Rats which had been on 4 per cent casein diet for 22 days and controls of the same age were divided into groups of 4 rats. To one test and one control group 1·0 ml. of a suspension of live *C. murium* containing 5×10^9 organisms was injected subcutaneously into the thigh. To the other groups 0·3 ml. of the same suspension was introduced intranasally. The results of this experiment

TABLE VI.—*Deaths from Infection with* C. murium *when Animals on 4 per cent Casein Diet and Controls on Stock Diet were Inoculated either Subcutaneously on Intranasally with Live Suspensions of the Organism.*

Dose of organism and route of infection.	Group of rats.	Time to death in days.
$5000 \times 10_6$ in 1 ml. s.c.	4 per cent casein diet	19, 19, 20, s
	Stock diet	s, s, s,* s*
1700×10^6 in 0·3 ml. i.n.	4 per cent casein diet	5, 14, (19†), 20
	Stock diet	8, s, s, s

s = Survived.
† Death probably not due to *C. murium*.
* Showed healing local abscesses at autopsy, but no progressive lesions.

are summarized in Table VI. The animals recorded as dying of pseudotuberculosis showed either progressive typical lung lesions or generalized pseudotuberculosis. Two of the control animals which had been inoculated subcutaneously showed small healing abscesses at autopsy, but none of this group showed progressive lesions, although 3 of the 4 protein-deficient animals died of the disease. In the groups given intranasal inoculations all the animals on the protein-deficient diet died, but one showed no evidence of infection with *C. murium*. One of the 4 controls died of typical pseudotuberculosis. If these groups are added together 6 of the 8 animals in the protein-deficient groups are found to have died of the disease and only 1 of 8 in the control groups.

Experiment 6.

Rats were divided into four groups of 6 and placed on diets containing 6, 8, 12 and 16 per cent casein. They were kept 16 days on the diet, during which time the average weight gain in the 6 per cent group was 2·5 g., in the 8 per cent 5 g., in the 12 per cent 6·7 g., and in the 16 per cent 10 g. (The expected weight gain for rats of similar age on 8 g. daily of stock diet would be 10–15 g.) At the end of this period all rats were inoculated intranasally with 0·2 ml. of a suspension of *C. murium* containing 2×10^9 organisms. The results are shown in Table VII. There does not appear to be any large or consistent difference in

TABLE VII.—*Deaths from Infection with* C. murium *when Animals on 6, 8, 12 and 16 per cent Casein Diets were Inoculated Intranasally with* 2×10^9 *Live Organisms.*

Group of rats.	Time to death in days.	Total number of specific deaths.	Number of rats in group.
6 per cent casein	3, 14, 21, 21, s, s	4	6
8 ,, ,,	4, 7, 16, 21 (21*), s	4	6
12 ,, ,,	4, 8 (21*), s, s, s†	2	6
16 ,, ,,	7, 7, 11, 11, s, s†	4	6

s = Survived.
* Death probably not due to pseudotuberculosis.
† Minimal healing lesions, but no progressive lesions at autopsy.

susceptibility between these groups, although the mortality of 2/6 in the group on the 12 per cent casein diet is lower than the 4/6 in all the other groups.

The serum taken from the surviving rats and from rats killed when moribund was pooled and tested for the presence of agglutinins against *C. murium*. Only low titre agglutinins were present in these pools, but the titre found in the group on the 6 per cent casein diet was lower than that found in the other pools (Table VIII).

TABLE VIII.—*Titre of Agglutinins against* C. murium *in Pooled Sera of Rats used in Experiment* 6 (*see text*).

Group of rats.	Number of sera in pool.	Agglutinin titre.
6 per cent casein	3	1:10
8 ,, ,,	2	1:40
12 ,, ,,	3	1:20
16 ,, ,,	2	1:20 ± 1:40

Experiment 7.

Groups of rats were put on 4 per cent casein diets at intervals of 14 days. Three groups of animals on low-protein diet and a group of controls on stock diet were inoculated with *C. murium* intranasally, receiving 2×10^9 organisms in 0·2 ml. The animals had either been on diet 28 or 14 days or had just been put on diet on the day on which they were inoculated. The results are shown in Table IX. There was very little difference in death rate between the groups of animals on diet for the different periods, but while 10 out of 18 animals on diet

TABLE IX.—*Deaths from Infection with* C. murium *when Animals* 28, 14 *and* 0 *Days on* 4 *per cent Casein Diet and Controls on Stock Diet were Inoculated Intranasally with* 2×10^9 *Live Organisms.*

Time on diet before inoculation.	Time to death in days.	Total number of specific deaths.	Number of rats in group.
28 days	7, 11, 16 (16*) s, s	3	6
14 ,,	7, 20, 21, 21, s, s	4	6
0 ,,	10, 12, 15, s, s, s	3	6
Controls	s†, s†, s†, s, s, s	0	6

s = Survived.
* Death probably not due to pseudotuberculosis.
† Minimal healing lesions, but no progressive lesions at autopsy.

TABLE X.—*Titre of Agglutinins for* C. murium *in Pooled Sera of Rats used in Experiment* 7 (*see text*).

Time on diet before innoculation	No. of sera in pool	Agglutinin titre
28 days	2	±1:10
14 ,,	4	1:10
0 ,,	3	±1:10
Controls	6	1:40

died, none out of 6 controls died, although 3 showed small healing lung abscesses at autopsy at the end of the experiment. The pooled sera of animals surviving to the end of the experiment or killed when moribund were tested for the presence of agglutinins for *C. murium*. Pools from the groups on 4 per cent casein showed agglutinins only to a titre of 1:10 or less, while the controls gave a titre of 1:40.

DISCUSSION.

The experiments presented above describe the results of a fairly close examination of a small number of animals. The results described in the first section show clearly that the most severe protein-deficient diet used had no effect on the titre of haemolytic complement in the strain of rats used. No evidence has been given on the effect on the various fractions of complement.

The results of the titration of agglutinins against bacteria do not give so clear-cut an answer. The differences in the titres against the *Salmonella* are negligible, but there is a slightly lower titre in the pooled sera of rats on a diet containing 4 or 6 per cent casein than in those of 8, 12 or 16 per cent casein or controls on stock diet, after they have been infected by intranasal inoculation of live cultures of *C. murium*. These results are comparable to those of Cannon, Chase and Wissler (1943), and Cannon, Wissler, Wooldridge and Berditt (1944), and lend some slight support to the view that the amount of protein in the diet may affect the ability to produce agglutinins demonstrable *in vitro*.

The results, however, strongly suggest that other factors than agglutinin production and complement titre are involved in resistance to infection. In the small experiment with *Salm. typhi-murium* there appears to be a difference in resistance between the immunized protein-deficient and control groups, although there is hardly any in their antibody titres, and the difference is comparable to the difference in resistance of the unimmunized animals.

In the case of the *C. murium* infections the course of the disease is usually slower and there is more opportunity for antibodies to develop. The difference between the animals on the 4 per cent casein diet and the controls on stock diet is clear cut. If all the results are pooled we find that 16 of 26 animals on the deficient diet died of the disease as against 1 out of 14 among the controls.

Bloomfield (1934) reported that rats on a protein-deficient diet similar to our own suffered a fall in serum protein of about 10 per cent during the first week on diet and that the serum protein was then stable. We had therefore thought it possible that the time on the diet might affect the result, but Experiment 7 gave no evidence that it had any effect in the case of *C. murium* infection. Experiment 6 showed that the difference between animals on the stock diet and those on the deficient diets was not due to protein deficiency. It appeared that the synthetic diet, reputedly having all the necessary factors in optimal amounts, was less capable of supporting resistance to infection than the commercial cube diet (see Webster, 1937).

The small differences in agglutinin titres between the different groups in Experiments 6 and 7 suggest, but certainly do not prove, that any differences in agglutinin titre between animals on protein-deficient and control diets may be due to the protein deficiency. If this is so it would appear that under the conditions of Experiment 6, 8 per cent casein was sufficient to allow maximal agglutinin formation. Although there was little difference in agglutinin titres between

animals on 8, 12 or 16 per cent casein and animals on stock diet, yet the former animals had a low resistance to *C. murium* very similar to that of animals on the protein-deficient diets, while the animals on stock diet were highly resistant to fatal infection with this organism.

SUMMARY.

1. The most highly protein-deficient diet used in these experiments has no effect on the production of haemolytic complement by rats.

2. There is some evidence of impairment of agglutinin production in rats on diets containing 4 or 6 per cent of casein, and rats on diets containing 8 per cent of casein or more produce agglutinins as well as those on stock diet.

3. Both before and after immunization, rats on 4 per cent casein diet were less resistant to *Salm. typhi-murium* than controls on stock diet.

4. Rats on 4, 6, 8, 12 or 16 per cent casein diet showed a greatly reduced resistance to fatal infection with *C. murium* compared to controls on stock diet, but there was no difference in resistance between groups on diets containing differing amounts of protein.

5. The time during which animals were on the diet did not affect the outcome of the experiment.

6. These experiments do not suggest that there is any close correlation between ability to produce agglutinins and resistance to fatal infection.

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EFFECTS OF LOW PROTEIN DIET ON THE MULTIPLICATION AND DISAPPEARANCE OF LOUPING ILL VIRUS IN THE RAT.

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THE experiments described in the preceding paper (Miles, 1951) confirmed our clinical impression that animals on the Himsworth diets were more susceptible to certain bacterial infections than normal animals. The results of MacCallum and Miles (1946) suggested that rats on such diets were more susceptible than normal rats to an agent which would pass through collodion membranes of 56 mμ. average pore-diameter and which was particularly active upon the liver parenchyma. The nature of this agent had not been elucidated, and it seemed unlikely that any further progress would be made with it unless light could be thrown on the problem of alterations in susceptibility using some other model in which the presence of the virus could easily be recognized. The experience of previous workers made us doubt whether we should find any great general alteration of susceptibility to virus infections, but we determined to try to find out what would happen in a suitable model.

Three viruses were known to give sub-clinical infection in the rat: influenza virus A (Hanford, Smith, McLeod and Wood, 1946), ectromelia virus (Burnet and Lush, 1936) and louping ill virus (Burnet, 1936).

None of these viruses was likely to prove in any way similar in action to the unknown agent found by MacCallum and Miles (1946), but we eventually decided to do a series of experiments using louping ill virus, because it can be passed easily to rats by intranasal inoculation, because it produces a very characteristic syndrome when inoculated intracerebrally into the mouse and because titrations in the mouse give sharp and easily-reproducible end-points.

At the time when this work was begun the only paper which we could find giving any mention of the multiplication of a neurotropic virus in animals on deficient diets was the short reference to the multiplication of Lansing poliomyelitis virus in the brains of thiamin-deficient mice given by Toomey, Frohing and Takacs (1944), but this work did suggest that the ability of virus to multiply might be separate from the ability to cause symptoms, and we therefore decided to investigate possible variations in the capacity of louping ill virus to multiply in the rat, using virus titrations in mice to follow the course of the infection.

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MATERIALS AND METHODS.

Rats.

The strain of rats used was bred in the laboratory starting from a single pair of albino rats. These rats have suffered from time to time from an acarine infestation which has proved extremely difficult to eradicate, but have otherwise been remarkably free from infection.

Mice.

In the earlier experiments most of the mice used were 5 weeks old miscellaneous mice brought in from one dealer. On several occasions experiments were completely ruined by intercurrent *Salmonella* infections, and in the later experiments mice of the same age bred in the laboratory from the Glaxo M.M. inbred strain were used. There was no obvious difference in susceptibility between mice from various sources when louping ill virus was inoculated intracerebrally, but in each experiment all mice used were from one source.

Virus.

A strain of louping ill virus was obtained as a dried suspension of sheep's brain from Dr. D. R. Wilson of the Moredun Institute, Edinburgh. This strain was passed three times intracerebrally in mice and then lyophilized. Dried virus from the 3rd to the 6th mouse passage was used in this series of experiments and did not show any alteration in its pathogenicity for mice. Mice infected with moderate or large doses of virus intracerebrally died on the 6th or 7th day. Mice infected with small doses died later, but there were very few deaths attributable to the virus later than the 12th day. All mouse titrations were carried through to the 14th day and surviving animals were then discarded.

Preparation of virus suspensions.

The lyophilized virus used in these experiments was prepared from the brains of mice dying on the 6th or 7th day after inoculation with moderate amounts of virus. At least 6 brains were pooled and ground without sand in a " Griffith " grinder with addition of a mixture consisting of 70 per cent horse serum and 30 per cent broth containing 7·5 per cent glucose. The suspension was made up to 10 per cent W/V with the diluent and subdivided into small tubes containing 0·25 ml. of fluid. These were dried from the frozen state. The average lethal dose (ALD) of the fresh material when inoculated intracerebrally into mice was normally 0·03 ml. of a dilution between 10^{-6} and 10^{-7}. During drying and the first 1–2 months following there was about a 10-fold fall in titre. Thereafter the titre remained constant for at least 10 months. (The first batch of dried virus was made up in 7·5 per cent glucose broth and suffered a much greater initial fall in titre than the later batches, although the titre remained constant after the initial fall.) The same batch of dried virus was used throughout each experiment.

For each experiment 2 to 4 tubes of dried virus were pooled to minimize variations. The virus was re-suspended in distilled water and then diluted in 10 per cent horse serum broth. In neutralization experiments 10 per cent horse serum saline was used instead.

Inoculation of mice and course of infection.

Mice were inoculated under ether anaesthesia with 0·03 ml. of suspension intracerebrally. The first sign of infection, with moderate doses of virus, appeared on the 5th or 6th day. Death nearly always occurred within 24 hours of the first sign. In these experiments as soon as unequivocal signs of infection were present the animal was killed.

Inoculation of rats.

Rats were inoculated intranasally under ether anaesthesia with 0·2 ml. of virus suspension. In the first experiment when the titre of the dried virus was low the rats were inoculated with about 7500 mouse ALD of virus. In all later experiments the dose was approximately 10,000 mouse ALD.

Harvesting material from rats.

Burnet (1936) reported that the virus multiplied in the nasal mucous membrane and the olfactory lobe of the brain of the rat, but did not normally spread further. Our preliminary observations confirmed this, but due to the convenience of being able, with reasonable care, to obtain sterile brain material, in all experiments the olfactory lobes of the brain plus a small area of adjoining cerebrum were removed. Early observations showed that in rats of the size we were using the olfactory lobes weighed about 0·1 g. Because of the variable amount of blood present and the convenience of being able to take a small but variable amount of cerebrum which did not contain significant amounts of virus, we have assumed in all experiments that the olfactory lobes of each rat used weighed 0·1 g. and all descriptions of dilutions are based on that assumption.

The rat brain was ground up with 10 per cent horse serum broth in Griffith's grinders except in Experiment 1, where individual olfactory lobes were ground with sterile sand in a test-tube by means of a glass rod. Suspensions extracted for 90 minutes at +2° were then centrifuged in an angle centrifuge at 3000 r.p.m. for 10 minutes. The supernatant was pipetted off and stored at +2° until used. Although Edward (1947) has shown that louping ill virus suspended in 10 per cent serum broth does not show any demonstrable fall in titre when kept overnight at 0°, all titrations were carried out the same day.

Neutralization tests.

a. *Test for presence of minimal antibody in individual sera.*—Virus was resuspended and diluted until 0·03 ml. of the suspension was estimated to contain 10 ALD of virus. To 0·2 ml. of this suspension 0·2 ml. of the test serum was added, so that in the final suspension 0·03 ml. contained 5 ALD of virus. Controls were set up containing 10 times the amount of virus in the test, the same amount and 1/10 the amount. The controls had pooled normal rat serum in place of the test serum. The mixtures were incubated for 90 minutes at 37° and then stored at +2° until used (usually about 2 hours). The experiments were so arranged that all tests had been mixed approximately the same time. Each specimen was tested on a group of 4 mice.

b. *Titration of pooled antibodies.*—Appropriate dilutions of virus were made in serum saline and distributed into 5 ml. bottles in 0·2 ml. quantities: 0·2 ml. was taken from each specimen of serum required for the pool and these equal

volumes were mixed : 0·2 ml. volumes from these pools were added to the volumes of virus. A control series contained pooled normal rat serum in the place of test serum. The processes of neutralization and inoculation were as described above.

Diets.

The diets used in this series of experiments were the same as those described in the previous paper except that a supplement of tocopherol was added, because it had been suggested by Himsworth, that, although it was not probable in so short a period, animals might develop a vitamin E deficiency during the course of our experiments (Himsworth and Lindan, 1949).

EXPERIMENTAL.

Experiment 1 : *Multiplication and Disappearance of Virus.*

For this experiment it was decided to attempt to demonstrate differences in the multiplication and disappearance of louping ill virus in the olfactory lobes of young rats in animals on the Himsworth 4 per cent casein diet and control animals on a similar weight of stock diet. Animals at the weight of about 70 g. were divided into groups so that from each litter there were equal numbers in the test and control groups. The animals were kept in groups of 6 in a cage and all were fed together. On the limited quantities of food, there were certainly some differences in the amount eaten by different rats. Such differences were largely due to the feeding habits of different animals, and made animals which appeared perfect litter pairs when first put into the cage develop very differently. Since it was not practicable to keep the rats in separate cages the exact paired litter-mate technique was not used in these experiments.

Animals which had been on test or control diet for 21 days were inoculated intranasally with approximately 2500 mouse ALD of louping ill virus under ether anaesthesia. After periods varying from 24 hours to 14 days the animals were killed, the olfactory lobes removed and kept separately. The olfactory lobes from each rat were then extracted as described above and titrated in mice. The numbers of mice used at each dilution and the dilutions tested varied with the time after inoculation and the number of mice available for use at the time.

The results show that virus was seldom present in the brain 24 hours after inoculation and normally it was present 48 hours after. The titre rose in both protein-deficient and control animals until the 6th day and then dropped abruptly, but much more rapidly in the control animals than in those on the deficient diet. The results are summarized in Tables I*a* and *b* ; in both Tables the numbers of rats are the same as shown in Table II. In Table I*a* the result shown was obtained by estimating the titre of virus in each rat using the Reed and Muench (1938) technique and taking the mean of the readings so obtained. In the few cases in which all the mice in all groups died it was assumed that the titre of virus was the lowest possible to correspond with the data available. That is that if all the animals in the highest group had died the titre was taken as half-way between that dilution and the next dilution above which should have been tested. This seemed to be a fairer method than neglecting all the unusually high figures.

In Table I*b* it was assumed that there were no substantial differences between

TABLE Ia.—*Comparison between Rates of Multiplication of Louping Ill Virus in Brains from Rats on 4 per cent Casein Diet and from Controls on Stock Diet.*

Days after intranasal inoculation.	Estimated mean virus titre in brain— From rats on 4 per cent casein diet.	From control rats.
1	3·3	0
2	70	49
3	300	100
4	2,440	1,960
5	14,430	5,090
6	30,420	31,330
7	2,400	380
8	4,260	335
10	1,800	42
14	0	0

TABLE Ib.—*Titration of Virus from Individual Rat Brains following Intranasal Inoculation of 2500 Mouse ALD: Comparison between Rats on 4 per cent Casein Diet and Rats on Stock Diet.*

Days after inoculation.	Proportions of mice dying after intracerebral inoculation of 0·03 ml. of the following dilutions of rat brain suspensions. From rats on diet. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	From control rats. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Virus titres in brain (reciprocal of dilution containing 1 ALD in 0·03 ml. of suspension). From rats on diet.	From control rats.
1	1/6					0/6					10	0
2	1/6	6/12				2/6	6/12				35	59
3	3/6		2/12			4/6		0/12			260	250
4	9/10	4/4	10/19	0/8		12/12	6/6	8/24	1/11		1,000	780
5	10/10	8/10	9/13	8/19		12/12	9/11	11/17	3/23		5,800	3,000
6	12/12	6/6	21/26	17/25	6/15	12/12	6/6	22/25	17/26	3/14	33,000	26,000
7	10/10	13/14	9/13	0/11		10/12	8/14	4/18	0/11		2,200	230
8	12/12	17/17	17/22	0/11		7/10	12/16	0/12	0/11		3,750	220
10	11/12	8/12	5/11			7/11	2/11	0/11			540	37
14	0/11					0/11					0	0

individual rats, and the results from all the mice used in each part of the experiment were added together and the same method was used to estimate the average lethal dose from the pooled mouse deaths. In the control animals on day 3 and in the test animals on day 5 and 10 these two methods give substantially different figures, but in general the differences between the titres estimated by the two methods are small, especially in view of the small numbers of mice used in the titrations.

The differences between the titres of test and control materials on the 7th and 8th and 10th days are significant. The differences on the other days are very slight, but there is a general tendency to find higher figures for the test than for the control animals.

The titres found in the individual rats are summarized in Table II. From this table it will be seen that there were rather more rats showing titres above

TABLE II.—*Comparison between Rates of Multiplication of Louping Ill Virus in Brains from Rats on 4 per cent Casein Diet and from Controls on Stock Diet.*

Days after intranasal inoculation.	Virus titres in individual rat olfactory lobes. From rats on 4 per cent casein diet.	From control rats.
1	1·0, 0, 0.	0, 0, 0.
2	2·0, 1·0, 2·0	1·0, 1·56, 2·0
3	0, 2·36, 2·83	1·7, 1·7, 0
4	3·0, 3·7*, 2·85, 3·7, 2·7	3·7*, 2·36, 2·36, 2·83, 3·36, 3·52
5	3·7, 4·0, 4·7, 1·7, 3·85	1·7, 3·7, 4·0, 4·0, 2·7, 2·7
6	4·0, 4·0, 4·7*, 4·92, 4·42, 3·38	4·0, 4·7*, 4·0, 4·7, 3·7, 4·8
7	3·7, 2·7, 2·7, 3·7*, 3·0	1·7, 1·7, 2·7, 3·0, 2·83
8	3·7, 3·7, 2·7, 3·7*, 3·7*, 3·7*	2·83, 2·5, 1·0, 0, 2·7*, 2·7*
10	3·7*, 1·83, 2·52	1·89, 1·7, 0.
14	0, 0, 0.	0, 0, 0.

* Animals for which the titre given is the minimum possible titre when the titration was not carried to the end-point.

The titres are expressed as the logarithm of the reciprocal of the dilution of rat brain which contained one mouse ALD of virus in 0·03 ml. of suspension.

the highest dilution tested among the animals on the low protein diet than among those on the control diet: hence full titration of all animals would probably have emphasized rather than reduced the differences which we find between the groups.

TABLE III.—*Development of Neutralizing Antibody against Louping Ill Virus in the Rat. Rats on 4 per cent Casein Diet and Controls on Stock Diet.*

Days after inoculation.	Number of sera in each group showing minimal neutralizing antibody. Diet rats.	Controls.
7	0(10)*	0(10)
8	0(7)	3(10)
9	0(10)	5(10)
10	0(10)	2(10)
11	6(10)	6(10)

* ( ) Number of rats in Group.

TABLE IV.—*Development of Neutralizing Antibody against Louping Ill Virus in the Rat. Rats on 4 per cent Casein Diet and Controls on Stock Diet.*

Days after inoculation.	Neutralization index of pooled rat sera. Diet rats.	Controls.
7	0(10)*	0(10)
8	0(7)	0(10)
9	0(10)	9(10)
10	0(10)	2·4(10)
11	3·6(10)	12(10)
14	160(10)	200(10)

* ( ) Number of rats in Group.

Development of neutralizing antibody.

Significant differences in the amounts of virus in the brains of the rats of the two groups were only apparent during the period in which the titre was falling. Although we had failed to show any close correlation between the formation of anti-bacterial agglutinins and resistance to bacterial infection, we investigated the relation between the development of neutralizing antibody and the rate of virus disappearance. It was felt that neutralizing antibody might show a closer relation to resistance than antibody demonstrable by agglutination.

Groups of 10 animals on 4 per cent casein and on stock diets were inoculated by the same technique with the same dose of virus and were killed on the 7th, 8th, 9th, 10th, 11th or 14th day and were bled out.

The presence of neutralizing antibody was estimated in two ways :

a. Serum from each individual rat was mixed with an estimated 5 mouse ALD of virus and the mixture after neutralization was tested by intracerebral inoculation into groups of four mice.

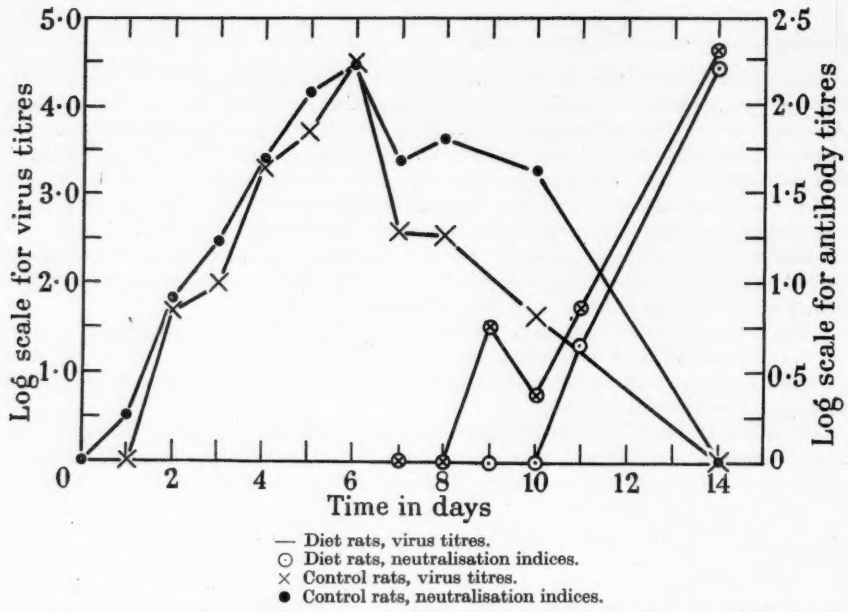
b. When the first technique suggested the presence of antibody in any of the sera, 0·2 ml. from each rat was mixed in a pool, and the neutralizing antibody present in the pooled sera was titrated using 10-fold dilutions of virus and groups of 5 mice for each dilution.

On the 14th day only the pooled sera were titrated, as it was assumed that all of the sera would contain recognizable amounts of neutralizing antibody.

The results are summarized in Tables III and IV. In the test for day 8, 3 of the animals on the low protein diet died on the 6th day for no obvious cause, although virus was present in fluid blood from the heart of one, and the cause of death may have been a breakdown of the constitutional barriers against the virus. There were therefore only 7 sera tested in this group. It will be seen from Table III that, from the 8th day onwards, some sera of the animals on stock diet showed antibodies ; while none of the animals on the 4 per cent casein diet showed any antibodies until the 11th day, when minimal antibody was present in 6 of 10 animals in each group. Table IV summarizes the results of titration of pooled sera from the groups in which individual sera showed antibody. In the control pools from 9th and 10th day animals low titre antibodies were present, and in the test animals there was a rather lower titre than in the controls on the 11th day. The titre in both groups has gone right up by the 14th day.

The figure shows the relation of all these results graphically. They strongly suggest that there is some delay in the formation of neutralizing antibody in the animals on a low protein diet, and that this delay is an important factor in allowing the virus to survive in the olfactory lobes of these animals.

It is possible to extract from later experiments, devised for other purposes, comparable figures for the multiplication and disappearance of virus in animals on the same two diets and also comparable figures for neutralization indices. In these cases the results were from pooled olfactory lobes or pooled sera only, and although the same batch of dried virus was used for each pair of experiments compared, the experiments were not necessarily carried out on the same day. The results are therefore not so strictly comparable as those described above. However, they are similar to those found in the first experiment and add confirmatory evidence (Tables V and VI). It is interesting that not only on the 10th day after inoculation, where the differences are very great, but also on the 3rd



Multiplication and disappearance of louping ill virus and the development of neutralising antibodies in rats on 4 per cent casein diet and stock diet.

TABLE V.—*Multiplication and Disappearance of Louping Ill Virus in Rat Brain: Virus Titres found in Different Experiments on 3rd, 6th and 10th days after Inoculation.*

Day.	Diet.	Titre in each experiment.			Mean*.
3	4 per cent casein	300	490		395
	Stock	100	220		160
6	4 per cent casein	30,420	588,000		309,210
	Stock	31,330	50,000		40,670
10	4 per cent casein	1,800	37	380	772
	Stock	42	<10	<10	14

* Mean figures for first experiment and pool figures in other experiments.

TABLE VI.—*Development of Neutralizing Antibody in Rats Infected with Louping Ill Virus: Summary of Neutralization Indices in Pooled Rat Sera on 7th and 10th days after Inoculation.*

Day.	Diet.	Titre in each experiment.			Mean.
7	4% casein	0(10)*	0(6)	0(6)	0
	Stock	0(10)	3·6(6)	5.3(6)	3·0
10	4% casein	0(10)	3·0(6)	5·0(6)	2·67
	Stock	2·4(10)	6·5(6)	4·0(6)	4·3

* ( ) Number of animals in group.

and 6th days where the differences were smaller, there appears to be a consistent tendency for the animals on the protein-deficient diet to have more virus in the olfactory lobes than the controls. In 8 groups of observations 6 have shown this tendency. In the remaining 2 (those for day 2 and day 6 of experiment 1—see Tables I and II) the figures have been estimated in two different ways, one giving a slightly higher titre in the animals on the deficient diet and the other a slightly higher titre in the controls.

Experiment 2 : *Comparison of* 4 *and* 16 *per cent Casein Diets.*

The findings of Experiment 1 showed that there was a significant difference in the rate of disappearance of virus between animals on a protein-deficient diet and those on the stock diet. The delay in disappearance of virus from the brain of the deficient animals could be correlated with a delay in the appearance of neutralizing antibody in their serum.

The experiments recorded in the previous paper (Miles, 1951) suggested that there were differences in agglutinin production between animals on protein-deficient and normal diet, and that although agglutinin production could be brought back to normal by increasing the amount of protein in a "synthetic" diet, yet the resistance to bacterial infection remained unchanged.

TABLE VII.—*Multiplication and Disappearance of Louping Ill Virus in Rat Brain : Comparison between Animals on Diets containing* 4 *and* 16 *per cent Casein.*

Days after Inoculation.	Titres from pooled olfactory lobes of groups of 6 rats— On 4 per cent casein diet.	On 16 per cent casein diet.
6	170,000	85,000
10	50	34
repeat 10	50	10

TABLE VIII.—*Titre of Neutralizing Antibodies on the* 10*th day after Inoculation with Louping Ill Virus in Rats on Diets containing* 4 *and* 16 *per cent Casein.*

Neutralization indices of pooled sera from groups of 6 rats— On 4 per cent casein diet.	On 16 per cent casein diet.
0	50
19	195

An experiment was therefore designed to show whether there was any great difference in the rate of disappearance of virus between animals on diets containing 4 and 16 per cent casein and whether antibody production differed between these groups.

Groups of 6 rats were kept on the diets for 3 weeks and then inoculated with approximately 10,000 mouse ALD of dried re-constituted louping ill virus in a volume of 0·2 ml. intranasally under ether anaesthesia. Groups were killed on the 6th or 10th days after inoculation. In all groups killed the olfactory lobes

were taken and those from each group pooled, extracted and titrated in groups of 5 mice. Blood was taken from the animals killed on the 10th day, and equal volumes of the sera from the members of each group were pooled and the pools were tested for neutralizing antibodies.

The results are summarized in Tables VII and VIII.

It will be seen that in each case the titre of virus in the animals on the 4 per cent casein diet was rather higher than that in those on the 16 per cent, but the differences were much less than those seen in Experiment 1 and were not significant. On the other hand, the difference in the titres of neutralizing antibodies was quite as great as that found in the former experiment, and the neutralization indices of the sera from the animals on the 16 per cent casein diet were the highest titres found in any animals bled on the 10th day, except for those to be recorded in a later paper.

Experiment 3 : *Adaptation of the Virus to the Rat.*

Two attempts were made to see whether passage of the virus in the rat could be carried on, and whether the virus could be adapted to the rat by using animals on either the 4 cent per casein diet or controls on stock diet or on 16 per cent casein diet.

TABLE IX.—*Passage of Louping Ill Virus in Rats.*

A. *Rats on* 4 *per cent Casein and Stock Diets.*

Passage	Day of death of inoculated mice. 4 per cent casein diet.	Stock diet.
1	6, 6	6, 6
2	6, s	7, 9
3	s, s	s, s

B. *Rats on* 4 *per cent and* 16 *per cent Casein Diets.*

Passage	Day of death of inoculated mice. 4 per cent casein diet.	16 per cent casein diet.
1	6, 6	6, 6
2	8, 11	s, s
3	s, s	s, s

s = Animals which survived 14 days.

Presence of virus tested by intracerebral inoculation to pairs of mice of a 10 per cent suspension of olfactory lobes harvested on the sixth day after intranasal inoculation of rats.

In each case 4 to 6 animals in test and control groups were killed on the 6th day after inoculation, and the pooled material was passed on the same day as a 10 per cent suspension of olfactory lobes 0·2 ml. intranasally under ether anaesthesia to further rats.

The presence or absence of virus was tested by injecting the same material intracerebrally into mice. No evidence of adaptation was obtained and no significant differences were found between the groups. The results are shown in Table IX. In the first experiment virus was present in the second passage material from the animals in both groups, but not in the third.

In the experiment using 4 and 16 per cent casein diets the virus was present in the second passage material in the groups receiving the 4 per cent casein only. On the third passage no virus was recovered from either group.

Discussion.

The results of Experiment 1 show that there is little difference in the rate of multiplication of louping ill virus in the olfactory lobes of the rat according to whether the animal is on a protein-deficient or a stock diet. Such evidence as is presented in Experiment 2 suggests that similar results can be obtained using diets in which only the protein is varied. Many investigators have examined the effects of dietary deficiency on the course of virus infections and, in the majority of cases, have found the deficient animals either equally resistant or more resistant than control animals; but extremely little work has been done on the effects of diet on the multiplication of virus in the host.

Such experiments as have been published (Toomey *et al.*, 1944, and Kearney *et al.*, 1948) on the multiplication of virus in animals on deficient diets which did not develop typical clinical disease as readily as controls do not give any evidence that virus multiplies less in the animals on the deficient diets than in those on control diets.

In our experiments the virus disappeared more slowly from the brains of animals on the deficient diet than those on the stock diet. The same trend was found in the animals on 4 and 16 per cent casein diet, but the differences were not significant.

Neutralizing antibody showed very suggestive alterations in its rate of development between the animals on deficient and on the stock diets, but such differences were even more marked between the animals on 4 and 16 per cent casein diets. The animals on the 16 per cent casein diets showed very high titres of neutralising antibodies on the 10th day. In nearly every case evidence has been presented that these results are repeatable.

There is an obvious correlation between the rate of antibody production and the rate of disappearance of virus from the brain, but the differences between the results obtained with the stock diet and those obtained with the 16 per cent casein diet, while they still allow that the development of neutralizing antibody is an important factor, suggest that it is not the only factor controlling the elimination of the virus.

Experiment 3 shows that, as one would expect, there were no significant differences between the groups in the ease with which virus could be passaged in rats. The virus did not adapt to the rat, and our evidence strongly suggests that there is no reason to anticipate that the use of the Himsworth type of diet will make it possible to adapt even those viral encephalitides which commonly show generalized infection in susceptible species.

Summary.

1. There are no significant differences in the rate of multiplication of louping ill virus in the olfactory lobes of rats on a 4 per cent casein diet or a stock diet. Less comprehensive results suggest that the same is true when 4 and 16 per cent casein diets are used.

2. There is a significant difference in the rate of disappearance of virus, the virus remaining at a higher titre in the animals on 4 per cent casein diet than in animals on stock diet. This correlates with a difference in the rate of development of neutralizing antibodies.

3. When 4 and 16 per cent casein diets are used the same tendency remains but the differences are no longer significant, although the antibody production of animals on the 16 per cent casein diet is at least as good as that of animals on stock diet.

4. It is concluded that the rate of development of neutralizing antibody is a factor, but not the only factor, controlling the rate of disappearance of virus from an infected tissue.

5. There is no significant difference between the ease with which virus can be passed from rat to rat in animals on the different diets. No more than two successful passages were achieved.

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STUDIES ON STAPHYLOCOCCUS MUTATION: A NATURALLY OCCURRING "G" GONIDIAL VARIANT AND ITS CARBON DIOXIDE REQUIREMENTS.

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SMALL colony variants have been described by many workers. They have been selected from cultures with the aid of inhibitory media, and a few have sometimes been seen among normal colonies on plates obtained from lesions. In the case reported a pure culture of a small colony variant staphylococcus was obtained. A patient of this hospital developed an abscess and culture of the pus on blood agar plates resulted in a pure growth of minute colonies, smears of which showed typical staphylococcal morphology. Plates from the same material incubated in the presence of CO_2 gave a growth of typical large staphylococcal colonies. This variant was not only isolated from the abscess, but was also shown to be carried in the patient's nose.

The effect of CO_2 in enhancing the growth of many bacterial species is well known, but its action as an essential factor for normal growth of a staphylococcus appears to be a new phenomenon. Furthermore, the isolation of a small colony variant in pure culture from a well-developed lesion raises certain points of both theoretical and practical importance. The organism was therefore investigated with a view to elucidating the role of CO_2 in the metabolism.

METHODS.

Preparation of bacterial suspensions.—The variant and "wild type" were grown on blood agar, both in air and in the presence of added CO_2. The growth was washed off the plates with physiological saline, centrifuged, washed, re-centrifuged and finally re-suspended in saline to give thick suspensions.

Estimation of dried weight of organisms.—Three aliquots of each thick suspension were pipetted into weighted tubes and an identical quantity of physiological saline (as used in making suspensions) was pipetted into another weighed tube. Tubes were dried at 100° to a constant weight, and the difference in weight between dried suspension and saline enabled the dried weight of the organisms used in the experiment to be calculated.

Measurement of oxygen uptake.—This was measured by the Warburg technique at 37°: 0·5 ml. of cell suspension, 0·5 ml. of substrate and 2 ml. of 0·158 M phosphate buffer, pH 7·2 were placed in the flasks. The final substrate concentration is given in the appropriate tables. Results are expressed as the QO_2 µl. of O_2 per mg. dried cells per hour.

Anaerobic glycolysis.—These measurements were performed at 37° using an atmosphere of N_2 containing 5 per cent CO_2, yellow phosphorus being placed in

the centre well of the Warburg flask to ensure complete anaerobiosis. For these experiments cells were suspended in the physiological salt solution described by Krebs and Henseleit (1932) and the method described by Dixon (1943) was followed. Results were expressed as the QN_2/G µl. CO_2 per mg. dried cells per hour.

Anaerobic dismutation of pyruvate and decarboxylations.—These were carried out by the method described by Smyth (1940). The gaseous phase was nitrogen, 5 per cent CO_2, anaerobiosis being ensured as in glycolysis experiments.

RESULTS,

Effect of CO_2.

Nutrient agar plates seeded with the variant and incubated aerobically for 24 hours yielded small colonies about 0·15 mm. in diamater. The use of blood agar resulted in an even smaller colony. Typical large staphylococcal colonies were obtained on both media after growth in the presence of CO_2, and they retained this large colony form through repeated subculture in these conditions. Reversion to small colonies followed immediately upon aerobic incubation. After 3–4 successive aerobic subcultures of the small variant a few large colonies appeared. These large colonies retained this form and were taken to represent what may be termed the " wild type " staphylococcus, that is, the type of organism from which the small variant was probably derived. Colony sizes of this " wild type " and other strains of staphylococcus were little affected by growth in CO_2; some showed very slight increases, others slight decreases. Incubation of the small colony variant in closed jars containing soda lime for the absorption of all traces of CO_2 resulted in even smaller colonies, whereas the wild and other strains of staphylococci were unaffected. In closed jars without soda lime colonies of the variant were slightly larger than colonies of ordinary aerobic cultures, owing to the accumulation of CO_2 from the organism's metabolism.

The experimental results are summarized in Table I.

TABLE I.—*Effect of CO_2 on Growth of the Variant and Other Staphylococci.*

Strain.	Mean diam. of colonies (mm.) when incubated under following conditions.			
	Air.	10% CO_2.	Closed jar.	Closed jar + soda lime.
Variant (Nutrient agar)	0·15	1·0	0·25	0·01
Variant (Blood agar)	0·05	1·0	—	—
Wild type	1·0	1·0	1·0	1·0
Albus	1·0	1·0	1·0	1·0
Aureus 18L	1·0	1·0	—	—
Aureus 35L	0·7	1·0	—	—
S. Newman	1·0	1·0	—	—
Aureus E	1·1	1·5	—	—
Aureus 709	0·9	0·9	—	0·9
Aureus 719	0·9	0·9	—	0·9

Medium unless otherwise stated is nutrient agar.

Effect of pH.

To test the possibility that CO_2 exerted its effect by an alteration of pH, nutrient agar plates with a pH range from 5·8 to 8·4 were poured. Duplicate plates were seeded with the variant; one incubated aerobically and the other in the presence of CO_2. Table II shows that over the whole pH range tested, CO_2 caused an increase in the colony size of the variant. It is, therefore, improbable that stimulation of the variant's growth by CO_2 is the result of pH changes.

TABLE II.—*The Effect of pH on the Growth of the Variant.*

Nutrient agar pH.	Mean diam. of colonies (mm.). Incubation in air.	Incubation in 10% CO_2.	Hours' incubation.
5·8	0·02	0·25	17
6·0	0·03	0·3	17
6·4	0·03	0·3	17
6·8	1·0	3·0	48
7·4	0·3	1·2	17
7·6	0·3	1·2	17
8·0	1·1	3·2	48
8·4	1·4	3·5	48

Attempts at replacement of the CO_2 requirement by other metabolites.

The use of enriched media, serum or yeast extract agar did not improve the growth of the variant. Pappenheimer and Hottle (1940) demonstrated an interesting relationship between adenylic acid and CO_2 tension. They found that a Lancefield Group A streptococcus required adenine or adenylic acid for growth, but that an absence of these factors could be offset by an increase in the CO_2 tension. In view of this finding, adenylic acid was added to media used for the growth of the variant, but it was without effect.

METABOLIC EXPERIMENTS.

Table III shows that with the substrates tested, the oxygen uptake of the variant is lower than that of the "wild type" when both have been grown aerobically. Growth in the presence of CO_2 has little effect on the oxygen uptake of the "wild type," whereas in the case of the variant, oxygen uptakes are restored to values that approach those exhibited by the "wild type." Thus it would appear that the variant strain, when grown in CO_2, elaborates enzyme systems identical with those of the "wild type," although subsequent culture in the absence of CO_2 results in a loss of one or more enzyme systems so that this abnormal small colony type of growth is resumed.

Krebs and Johnson (1937) have shown that fumarate stimulates the respiration of certain tissues through the agency of a tri-carboxylic acid cycle. When pyruvate is used as the substrate, fumarate stimulates the oxygen uptake both of the "wild type" and the variant grown in CO_2, but is without effect in the case of the variant grown in air.

TABLE III.—*Oxygen Uptake of the Variant and "Wild Type."*

Substrate.	QO_2 of suspensions. Variant grown in 10% CO_2.	Variant grown in Air.	"Wild type" grown in 10% CO_2.	"Wild type" grown in Air.
Glucose 0·025 M	−157·5	−57·9	−178·8	−144·6
Peptone 0·1%	−206	−117·2	−220·4	−197·2
Pyruvate 0·043 M	−82·8	−33	−116	−111·7
Fumarate 0·043 M	−40·1	−11·7	−41·6	−39·9
Pyruvate 0·043 M + Fumarate 0·043 M	−136·3	−35·5	−152·9	−147·5

Dismutation of pyruvic acid.

Krebs (1937) demonstrated in staphylococci a dismutation of pyruvate to lactate and acetate with the liberation of CO_2. Table IV demonstrates the relative capacity of the variant when grown in air to carry out this reaction. After growth in CO_2 this capacity was restored. Some increased activity was also observed with the "wild type" grown in CO_2. Lipmann (1937) and Hills (1938) studied this reaction in *Lactobacillus delbrückii* and staphylococci and found that co-carboxylase (a derivative of Vitamin B_1), adenine dinucleotide, magnesium or manganese ions and inorganic phosphate were required as co-factors. The addition of vitamin B_1 to suspensions of the variant did not stimulate pyruvic acid dismutation, therefore there was probably no deficiency of co-carboxylase Yeast extract added to the reaction mixtures increased the velocity of the dismutation, both with the variant and the "wild type." Although the velocity increased in both cases, the difference between the variant grown in air and in the presence of CO_2 remained obvious. In preparation of cell suspensions, coenzymes and accessory factors are probably lost during the washing, so that their replacement by the addition of yeast extract would account for the general stimulation. The loss of activity shown by cells stored in the refrigerator is also restored by the addition of yeast extract. Thus it appears that the variant grown in air is deficient in the actual apoenzyme and not in coenzymes or accessory factors.

TABLE IV.—*Anaerobic Dismutation of Pyruvic Acid and Decarboxylation of Oxaloacetic Acid by the Variant and "Wild Type" Staphylococcus.*

Substrate.	QCO_2 of suspensions. Variant grown in 10% CO_2.	Variant grown in Air.	"Wild type" grown in 10% CO_2.	"Wild type" grown in Air.
Pyruvate 0·172 M	+28	+15·7	+40	+31
Oxaloacetic 0·172 M	+14·7	+4	+10·6	+9·6

Decarboxylation of oxaloacetate and α-ketoglutarate.

Krampitz and Werkman (1941) isolated an enzyme from *Micrococcus lysodeikticus* that decarboxylated oxaloacetate to pyruvate. This enzyme, working in the reverse direction, was considered by many workers to be responsible for

CO_2 fixation in organisms (Wood and Werkman, 1935, 1936, 1940*a*, *b*; Werkman and Wood, 1942; Wood *et al.*, 1941). Both the variant and the "wild type" decarboxylated oxaloacetate, but, as Table IV shows, the same differences are seen as when pyruvic acid is decarboxylated. The decarboxylation of α-ketoglutarate is one of the reactions in the tricarboxylic acid cycle postulated by Krebs and Johnson (1937), but both the variant and "wild type" failed to decarboxylate this compound.

Inadequate utilization of pyruvate by variant strain.

The anaerobic glycolysis of the variant, whether grown in air or in the presence of CO_2, was found to be the same as that of the "wild type." Pyruvic acid production should be the same when the suspensions of the variant, grown either in air or in 10 per cent CO_2, are incubated with glucose. As the subsequent dismutation of the pyruvic acid would be slower with cells grown in air, it should be possible to demonstrate a greater accumulation of pyruvic acid than with cells grown in CO_2. In order to test this hypothesis the variant staphylococcus was seeded on blood-agar plates, some of which were incubated in air and others in CO_2. After harvesting in the usual way, 0·5 ml. of each suspension was pipetted into flasks containing 9·5 ml. of 0·25 per cent glucose in isotonic phosphate buffer pH 7·2, which were incubated for 1 hour at 37°. At the end of this period 0·5 and 1·0 ml. samples were removed from each flask, the cells removed by centrifuging and the keto acids present in the samples estimated by the technique described by LePage (1950). In the mixture containing the variant grown aerobically, 3 μg. pyruvate per mg. of dried cells was detected. But when CO_2 was used, no trace of pyruvate was found. In neither instance was oxaloacetate or α-ketoglutarate detected in the samples.

DISCUSSION.

The small colony variant described was isolated in pure culture from a typical staphylococcal lesion and was also carried in the patient's nose. It is therefore probable that conditions in the body gave the variant a survival value over the parent strain. The case reported is probably not an isolated example, and the use of antibiotics may well provide selective conditions for a wide range of small variants. The growth of such variants, however, is so scanty that they could easily be missed in routine cultures from lesions where they were the sole invading organisms, unless the possibility of their existence were borne in mind.

The experiments reported above show that the small variant has a diminished capacity to bring about the dismutation of pyruvic acid and decarboxylation of oxaloacetate. When the variant is grown in the presence of CO_2 these capacities are restored to normal.

Smyth (1940), working in Krebs' laboratory, confirmed the work of Hills (1938) that vitamin B_1—deficient staphylococci exhibited a diminished capacity for pyruvic dismutation. He further demonstrated that this reaction could not only be restored by the addition of vitamin B_1, but also by oxaloacetate or fumarate. The function of the oxalocetate or fumarate was, he thought, to act as a carrier:

$$\text{Pyruvate} + \text{Oxaloacetate} \longrightarrow \text{Malate} + \text{Acetate}$$
$$\text{Pyruvate} + \text{Malate} \longrightarrow \text{Lactate} + \text{Oxaloacetate},$$

the function of the vitamin B_1 being to catalyse the reaction,

$$\text{Pyruvate} + \text{Carbon Dioxide} \longrightarrow \text{Oxaloacetate},$$

and thus supply the carrier.

Addition of oxaloacetate to the pyruvate failed to restore the ability of the variant grown in air to bring about the dismutation of the pyruvate. The failure to restore activity by use of these compounds or the addition of known coenzymes is very suggestive that the deficiency is one of the apoenzymes.

Smyth (1940) was of the opinion that the tricarboxylic acid cycle did not function in the staphylococcus, but that there was a modified Szent Gyorgyi cycle in operation. The results presented confirm this view since no decarboxylation of α-ketoglutarate could be demonstrated. Smyth apparently believes that the same enzyme functions both in the dismutation of the pyruvate and in the production of the oxaloacetate. On the other hand Krampitz and Werkman (1941) suggest that the enzyme bringing about the dismutation of pyruvate is quite distinct from the enzyme they obtained, which decarboxylated oxaloacetate. This enzyme, working in reverse, is, they state, responsible for carbon dioxide fixation and oxaloacetate production.

It is possible to advance a tentative hypothesis that will account for the finding that, after growth in the presence of carbon dioxide the variant cells have the same enzymic content as the "wild type." The increase in carbon dioxide tension will probably facilitate the reaction,

$$\text{Pyruvate} + CO_2 \longrightarrow \text{Oxaloacetate},$$

by swinging it in favour of oxaloacetate production. In so doing the enzyme concerned may be adaptively increased and, accepting the view of Smyth that this enzyme is also concerned in the dismutation of pyruvate, then this (metabolic mechanism) will also be restored. As the capacity to produce this enzyme is dependent on an increased carbon dioxide tension, the stimulus to production of the enzyme will cease to operate under aerobic conditions, so that the cells will again become deficient.

The normal staphylococcus grown in air must be able to synthesize adequate amounts of the enzyme or enzymes postulated. In the case of the variant, the genetic control of the enzymes may be altered in such a fashion that enzyme synthesis is impaired, but still capable of adaptive increase under the stimulus of carbon dioxide. If the view of Krampitz and Werkman that there are two different enzymes involved, one decarboxylating oxaloacetate and the other causing pyruvate dismutation, is correct, then the above reasoning explains only the restoration of oxaloacetate decarboxylation. Since this work was completed, however, Watt and Werkman (1950) have shown that the reaction,

$$\text{Pyruvate} \longrightarrow \text{Acetate} + \text{Lactate} + CO_2,$$

is reversible and is a method of CO_2 fixation in the staphylococci. The hypothesis postulated can equally well be applied to this reaction.

SUMMARY.

1. A small colony variant of staphylococcus isolated in pure culture from an abscess was found to give the normal large colony type of growth in the presence of 10 per cent CO_2.

2. This variant was found to bring about dismutation of pyruvate much more slowly than did normal staphylococci, and addition of coenzymes and accessory factors did not restore this ability.

3. After growth in carbon dioxide pyruvate dismutation proceeded normally, and therefore the cells must build up a full enzymic content in the presence of this gas, but fail to do so in its absence.

4. The dismutation of pyruvate is also a reaction by which CO_2 is fixed, and it is tentatively suggested that increased CO_2 tension favours this reverse reaction and in so doing results in an adaptive increase of the enzyme concerned.

5. The significance and the implications of the isolation from a patient of a small colony variant in pure culture are discussed.

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The author also wishes to express his indebtedness to Professor Wilson Smith for constant advice and criticism.

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THE ACTION OF PROTEOLYTIC ENZYMES ON THE ANTITOXINS AND PROTEINS IN IMMUNE SERA.

III. Further Studies on Enzyme Systems which Split the Antitoxin Molecule.

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In earlier papers (Pope, 1938, 1939*a*, *b*) results were presented showing the action of proteolytic enzymes on antitoxic sera. Additional work had been carried out in 1939 but was not completed owing to the outbreak of war, and only recently has it been possible to complete and extend this work.

The purpose of the present paper is to show in more detail the manner in which enzymes that split the antitoxin molecule act, and to present evidence which suggests that in pepsin, trypsin, papain, rennin, etc., there is present a special enzyme which has this function.

Experimental.

Methods.—Protein determinations and antitoxin estimations were made as described previously (Pope, 1939). For most of the work diphtheria antitoxic sera were used in the form of oxalated plasma, but in some cases a special insoluble substrate, described in detail later, was used. The enzymes employed are dealt with in detail as the work with them is described.

Optimal pH for Enzyme Action.

Experiments with pepsin were carried out in the following manner : Diphtheria antitoxic plasma, diluted with two volumes of water, was adjusted to pH values of 3·2, 3·6, 4·0 4·5, 5·0, 5·5, 6·0, 6·5 and 7·0, and to each 100 ml. 20 mg. of pepsin (1/3000 activity) was added. These samples were then heated to 37° for 24 hours, after which they were adjusted to pH 4·3 and 14 per cent ammonium sulphate (w/v) added. After filtration the antitoxin content of each filtrate was determined. In order to determine whether the antitoxin had the property of heat stability previously described (Pope, 1938, 1939) portions of the filtrates were heated to 60° for one hour. This heating caused precipitation of protein, and after filtration the antitoxin values were again determined. Results for this experiment are shown in Fig. 1. The antitoxin values in the filtrates, before heating, show an obvious effect of pH ; at values more acid than pH 5·0 there is a rapid fall in the antitoxin values. After the heat treatment the curve shows an optimal pH value for survival of the antitoxin at about 3·75 ; at pH 4·5 only 5 per cent of the antitoxin remains after heat treatment.

The action of pepsin is, as might be expected, restricted to the acid side, and it was therefore a matter of some interest when it was found that crude trypsin behaved similarly.

Fig. 2 shows results for an experiment in which Liq. Trypsin Co. (Allen & Hanbury's) was used. Conditions generally were as for the previous experiment except that the pH range was 3·2, 3·4, 3·8, 4·2, 4·5, 5·0, 6·0, 7·0, 5 per cent Liq. Trypsin Co. added to each, and the solutions were held for 4 days at 20° before

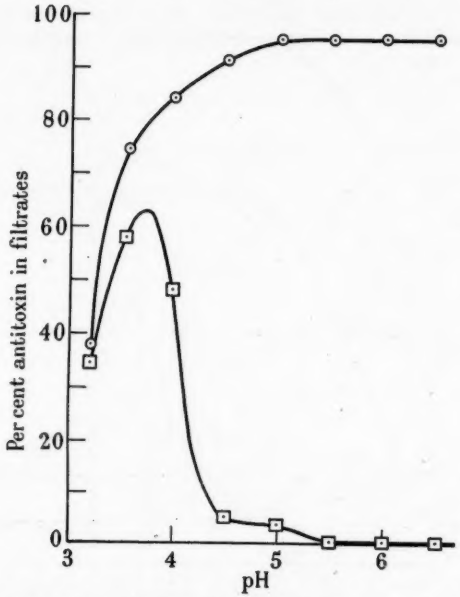

FIG. 1.—The action of pepsin (20 mg./100 ml.) on diphtheria antitoxin at varying pH values for 24 hours at 37°. All samples made to 14 per cent w/v ammonium sulphate and pH to 4·3 after incubation.

⊙——⊙ Per cent antitoxin in filtrates before heating.
⊡——⊡ Same after heating for 1 hr. at 60°.

adjusting to pH 4·3 and adding 14 per cent ammonium sulphate (w/v). After filtration the antitoxin value and protein content were determined (curves A and B, Fig. 2). Portions of each filtrate were heated to 60°, but in this experiment the time of heating was limited to 20 minutes. After filtration the antitoxin value and protein content were again determined (curves C and D, Fig. 2). Once again the maximal antitoxin stability on heating to 60° was at about pH 3·75. Curve D also shows that the soluble protein in the heated filtrates follows that of the antitoxin and is maximal at the same pH value. Experiments similar in nature were also carried out with crude papain (without activation) and with commercial rennin powder. In each case the pH for optimal activity was the same as for pepsin.

These results suggested that it would be a matter of interest to examine crystalline enzymes, and samples of crystalline trypsin and chymotrypsin were obtained (our thanks are due to Dr. J. Northrop for these materials). A modified method was used for the examination of these enzymes. The diphtheria antitoxic plasma, diluted with two volumes of water, was adjusted to pH 3·8, and either crystalline trypsin or chymotrypsin added in the quantities shown in Fig. 3. These amounts relate to the enzymes as supplied, and inert salts account for about 50 per cent of the weight; the actual quantities of enzyme proteins are therefore

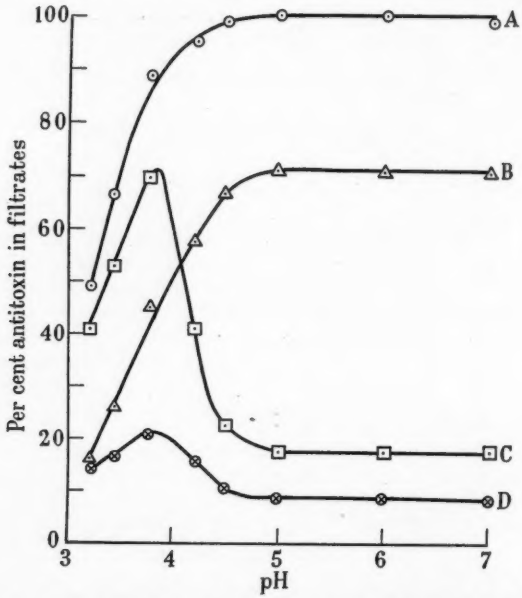

FIG. 2.—The action of crude trypsin on diphtheria antitoxin at varying pH values (5·0 ml. A & H trypsin/100 ml.). Kept for 4 days at 18°; then 14 per cent w/v ammonium sulphate and all adjusted to pH 4·3. Part filtered and remainder heated to 60° for 20 minutes.

A—Per cent antitoxin in filtrates (non-heated)
B—Per cent protein in filtrates (non-heated)
C—Per cent antitoxin in filtrates (heated)
D—Per cent protein in filtrates (heated)

about half those shown. After heating to 37° for 24 hours, 14 per cent (w/v) ammonium sulphate was added and the mixtures filtered. From the results in Fig. 3 it will be seen that in the absence of enzyme only a small percentage of the original antitoxin was found in the filtrates (about 10 per cent). Crystalline chymotrypsin was active in liberating antitoxin from the insoluble substrate, while crystalline trypsin was considerably less active. The characteristic increase in soluble protein is also shown. The antitoxin splitting activity of these crystalline enzymes at pH 3·8 was surprising, since this pH is far removed from that at which they are optimally active against substrates such as denatured haemoglobin.

We have also examined enzyme extracts prepared from ox and sheep pancreas. Using ox pancreas a preparation was made in the following way :

Minced ox pancreas (2000 g.) was diluted to 10 litres with water and allowed to stand for 24 hours at 4° ; 10*N* HCl was then added to adjust the pH to 3·6, and the product filtered. Each litre of filtrate was precipitated by adding 250 g. of ammonium sulphate and the precipitate discarded. The filtrate was again precipitated by adding a further 250 g./litre ammonium sulphate and the precipitate collected by filtration. It was dissolved in water to give a volume equal to 1/10 of the original. This solution was found to contain 0·37 per cent of protein.

Using this enzyme preparation, tests for enzyme activity were made as described for crystalline trypsin and chymotrypsin. The results obtained are

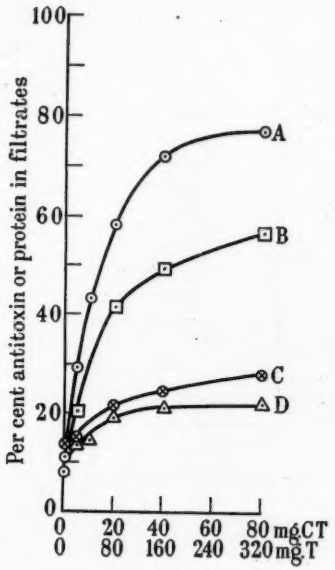

FIG. 3.—The action of crystalline chymotrypsin (CT) and trypsin (T) (Northrop) on diphtheria antitoxin at pH 3·8–24 hrs. at 37°. Liberation of antitoxin by increasing amounts of chymotrypsin A and trypsin B. Liberation of protein by increasing amounts of chymotrypsin C and trypsin D.

shown in Fig. 4. From these results it is clear that very much smaller quantities of enzyme protein from pancreas were needed for the liberation of antitoxin from the insoluble substrate than was the case with either crystalline trypsin or chymotrypsin. This preparation was not a pure enzyme ; it is therefore clear that even smaller quantities of pure enzyme would be required to liberate the antitoxin from the substrate. From this observation it appeared that the crystalline trypsin and chymotrypsin might be considered as having, as a contamination, a small amount of another enzyme which was active at pH 3·8 against the antitoxin substrate.

A further study was therefore made on this enzyme, using sheep pancreas which was available in considerable quantity. The isolation of the enzyme fraction and the methods used for assay are described below.

Isolation of the Antitoxin-splitting Enzyme from Sheep Pancreas.

Frozen sheep pancreas (1000 g.) was minced and extracted with cold N/4 H_2SO_4 (4·0 l). After straining through muslin the liquid was adjusted to pH 6·0 and re-filtered with the aid of Hyflo Supercel on a Buchner funnel. The

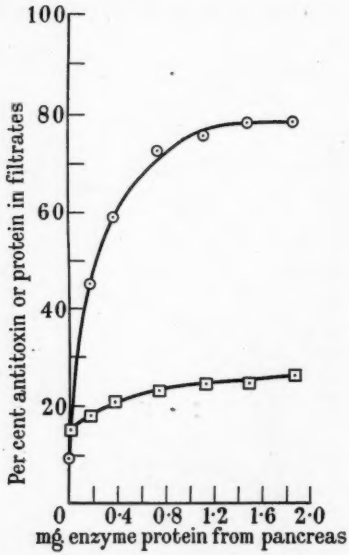

FIG. 4.—The action of an enzyme isolated from pancreas on diphtheria antitoxin at pH 3·8 and 24 hours at 37°. ⊙ per cent antitoxin and ⊡ per cent protein solubilised by increasing amounts of enzyme.

protein in the filtrate was precipitated completely with ammonium sulphate, and after separation by filtration, dissolved in water, dialysed to remove excess ammonium sulphate, and the volume then made to 500 ml. After adjusting the pH to 4·0 the protein was precipitated fractionally to yield six fractions as shown in Table I. Each fraction, after dialysis, was adjusted to a volume of 100 ml. and the protein content of each determined. The total protein present in the six fractions was 1·753 g. derived from 1000 g. of pancreas.

For the purpose of assaying the enzyme activity of these fractions the following method was used: Diphtheria antitoxin plasma was fractionated with ammonium sulphate and the protein precipitating between 1/3 and 1/2 saturation was isolated. The antitoxic protein was dissolved in water (giving a value of about 120 units per ml.), 8 per cent (w/v) ammonium sulphate added and the pH adjusted to 3·8. After heating to 55° for 1 hour the antitoxin became

insoluble and tests on a filtered portion showed the filtrate to be antitoxin-free. This substrate when acted upon by a slight excess of a suitable enzyme (pepsin or trypsin fraction) gave a maximal liberation of 80 units/ml. of antitoxin. As an arbitrary enzyme unit we selected the amount of enyzme which would liberate 30 units of antitoxin from this substrate in 24 hours at 37°. This was on the linear part of the curve relating the square root of the enzyme concentration with the units of antitoxin liberated under these conditions. Table I shows the results obtained in this experiment.

TABLE I.—*The Fractionation with Ammonium Sulphate of the Antitoxin-splitting Enzyme from Sheep Pancreas.*

Fraction.	Ammonium sulphate g./litre.	Volume of enzyme ≡ 1 unit. ml.	Enzyme units per ml.	Per cent of total enzyme present.
C1	0–100	0·0484	20·6	1·35
C2	100·150	0·0484	20·6	1·35
C3	150·200	0·0056	178·5	11·6
C4	200–250	0·0009	1111·0	72·5
C5	250–300	0·0056	178·5	11·6
C6	300–350	0·0484	20·6	1·35

The volume of enzyme solution equivalent to 1 unit was determined from a number of experiments in which the amount of antitoxin liberated from the substrate was both slightly more and slightly less than 30 units per ml., and the volume required to liberate 30 units found by interpolation. It will be seen that most of the enzyme was precipitated between 200 and 250 g. of ammonium sulphate per litre, and this was confirmed in a series of experiments. Many attempts were made to isolate the enzyme present in this fraction in crystalline form but without success.

Since the splitting of the antitoxin molecule gives no information about other enzymes present in this fraction we have examined its activity on haemoglobin at a series of pH values. The method used (Pope, Fenton and Jones, unpublished) is based on that of Anson (1938), except that crystalline horse haemoglobin (undenatured) was used as substrate, and the method used to produce the colour in the digestion products with the Folin-Ciocalteu phenol reagent (Folin and Ciocalteu, 1927) was modified in order to obtain more stable colours. A detailed account of this method and work on the fractionation of enzymes will be published elsewhere. Our choice of undenatured crystalline haemoglobin as substrate was made because the undenatured haemoglobin is less readily attacked by trypsin and chymotrypsin, and we wished to determine the degree of proteolysis in the acid pH zone. Fig. 5 shows the results obtained on examination of the active enzyme fraction in this way. The haemoglobin substrate was added to buffer solutions, and the pH values adjusted in steps of 0·25 pH unit across the range 2·0 to 9·0. To each mixture was added 0·5 ml. of a 1/20 dilution of the enzyme fraction C4 (see Table I); digestion was allowed to proceed for 4 hours at 40°. Excess substrate was then precipitated with trichloracetic acid (5 per cent final concentration), and after standing for at least 4 hours (or overnight) the mixtures

were centrifuged ; equal volumes from each mixture were removed and the colour was developed with the Folin-Ciocalteu phenol reagent. The colours were measured with a photoelectric colorimeter and, after correction for blank values, are shown in Fig. 5 as optical density (log 100–log per cent T).

From this curve it will be seen that this enzyme showed peak activity at about pH 3–3·5 together with an increasing activity from about pH 6–9, which would have been much greater with denatured haemoglobin. Fig. 5 also shows the activity, determined in the same way, for samples of crystalline trypsin and

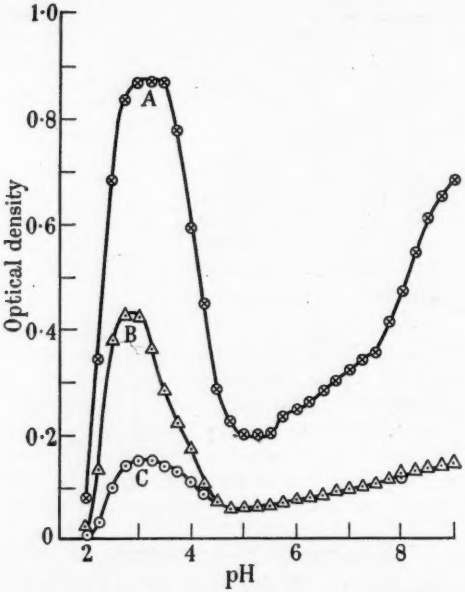

FIG. 5.—The action of an enzyme from sheep pancreas A, crystalline trypsin B, and crystalline chymotrypsin C on crystalline horse haemoglobin at different pH values. Optical density = Density of reading—Density of blank.

From pH 4·75 to pH 9·0 the values for curves B and C were identical except at pH 8 where there was a slight difference as shown in the curve.

crystalline chymotrypsin, both of which were obtained through the kindness of Dr. Taylor. These samples had been crystallized several times as trypsin or chymotrypsinogen, but both show the presence of an enzyme with peak activity at about pH 3. As a result of this finding the crystalline trypsin and chymotrypsin obtained from Dr. Northrop, together with another sample of crystalline trypsin obtained from Dr. E. Work, were examined for their ability to digest undenatured crystalline haemoglobin at pH 3·8. Unfortunately there was insufficient of these materials to do a full test at a series of pH values. However, they all showed rapid digestion of haemoglobin at pH 3·8, and it is possible that on an extended pH range they would have shown results similar to those shown in Fig. 5.

These observations on the enzymes extracted from pancreas suggest that there is present an enzyme with an optimal activity at pH 3–3·5, which can cause both the splitting of the antitoxin molecule and digestion of haemoglobin. The low activity, in terms of antitoxin splitting ability, of crystalline trypsin and chymotrypsin would be explained by the fact that these crystalline enzymes are contaminated with traces of this enzyme active at acid pH values.

Where the enzyme used for antitoxin-splitting was pepsin, it had been assumed that the enzyme responsible was in fact "pepsin" working at a pH value more alkaline than the optimal pH, but in view of the results with pancreatic

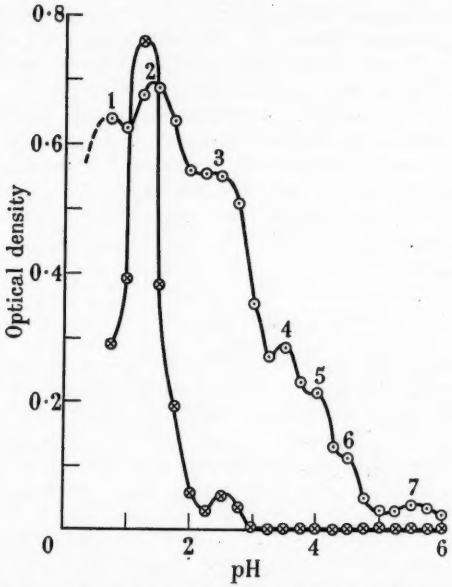

FIG. 6.—The action of pepsin (1/10,000 activity) and a fraction isolated from it on crystalline horse haemoglobin. Optical density = Density of reading − Density of blank. At pH 3·8 the antitoxin molecule is split by the crude pepsin but not by the fraction isolated from it.

extracts it was considered necessary to re-examine pepsin. Starting with pepsin with an activity of 1/10,000 we prepared from it twice crystallized material using the alcohol method of Northrop (1946). The two products were then compared on a weight basis for their ability to digest haemoglobin using a method based on that of Anson (1938). In agreement with Northrop, Kunitz and Herriott (1948) it was found that the crystalline pepsin was five times more active than the starting material. We then assayed these two preparations for their ability to liberate antitoxin from an insoluble substrate using the method described for trypsin. Once again the crystalline pepsin was found to be five times as active on a weight basis as the 1/10,000 pepsin. On this evidence it did not appear that the crystallization process had modified the ratio of activity at pH 1·5 to that at 3·8.

However, it was felt that other methods might throw some light on this problem and we therefore examined the action of pepsin on haemoglobin at a close series of pH values, using the method already described for the pancreatic enzymes. Both the crude 1/10,000 and the crystalline pepsins give curves of the type shown in Fig. 6A, where there is evidence for a series of enzyme-pH peaks (numbered 1–7). Here the pH-activity peaks numbered 4 and 5 might be responsible for splitting the antitoxin molecule, and much work has been done in an attempt to isolate enzymes with activity limited to this pH zone (3·5–4·0). So far we have had little success in this direction, but we have been able to isolate a fraction, by a method to be dealt with in detail elsewhere, with the activity shown in Fig. 6B. This fraction, corresponding to enzyme No. 2 in curve A, is slightly contaminated with enzymes No. 1 and 3, but is inactive at pH values more alkaline than pH 3. It was obviously a matter of interest to test fractions of this type for their ability to split antitoxin at pH 3·8. At the time these fractions were available we had no suitable diphtheria antitoxin substrate, but with the help of Dr. C. L. Oakley the tests were done on *Cl. welchii* antitoxin. These fractions produced virtually no splitting of the antitoxin at pH 3·8, whereas the controls with the original pepsin behaved normally. There appears therefore to be evidence that pepsin contains more than one enzyme ; one of these is concerned with the splitting of antitoxin at pH 3·8.

DISCUSSION.

Evidence has been presented to show that in the case of pepsin, and the enzyme extracted from pancreas, there is an apparent optimal pH at which the enzymes act upon antitoxic protein and split the molecule into two parts, one non-antitoxic and easily denatured, the other carrying the antitoxin and less readily denatured. This explanation of the effect of the enzymes was first suggested by Pope (1938), and subsequently confirmed by Petermann and Pappenheimer (1941). At a temperature of 38° this optimum appears to be at pH 3·8, but at lower temperatures there is evidence that the splitting takes place more rapidly at a pH of 3·2. The apparent variation with temperature is due to the destructive action of acids on antitoxin at more acid pH values at higher temperatures.

Although not dealt with in detail in this paper we have found that papain, rennin, crude maltase, an ultrafiltered concentrate from *Cl. welchii* toxin, etc., all appear to contain an enzyme system optimally active at pH 3·8 and 37°. These are now being investigated in more detail.

An examination of the two major enzymes from pancreas, which are active at alkaline pH values, showed that both crystalline trypsin and chymotrypsin possessed the property of splitting antitoxin at pH 3·8. The amount of enzyme protein required was rather large, and in the two samples examined the chymotrypsin was more active than the trypsin. The sample of chymotrypsin was labelled " 9 × chymotrypsin " and the trypsin " 3 × trypsin," and it is understood that this referred to the number of times the preparation had been crystallized. Our observation that a crude protein fraction isolated from pancreas was very much more active suggested that the crystalline enzymes were slightly contaminated with another enzyme which was responsible for the action on antitoxin at pH 3·8. Further investigation of these enzymes, using crystalline horse haemoglobin as

substrate, at close pH intervals showed that they were active on the acid side with a pH optimum at 3·0–3·5. This activity shown by the crystalline enzymes was the same as that of a fraction isolated from pancreas, assayed in terms of its ability to split antitoxin. From this work it appears that amongst the enzymes extractable from pancreas there is one with the property of splitting antitoxin, and that this is not a property of either trypsin or chymotrypsin except for the trace of enzyme with which they are contaminated. The fact that all samples of crystalline trypsin or chymotrypsin examined have some ability to split antitoxin and digest crystalline haemoglobin at pH 3·8 gives support to the view that the crystallinity of a protein is no guarantee of its purity.

The enzyme pepsin shows a similar apparent optimum at pH 3·8, and our investigations suggest that here again the action is not due to true pepsin, but to another enzyme active at more alkaline pH values. A careful examination of the action of both crude and twice crystallized pepsin at a series of close pH values against crystalline haemoglobin substrate suggests that there may be a series of enzymes present. Unfortunately we have not yet succeeded in isolating material with its activity limited to the necessary pH range, but we have isolated a fraction with its activity sharply limited to pH 1·25 with an absence of activity at pH 3 or higher. This fraction was found to be virtually inactive when tested for its ability to split antitoxin at pH 3·8. In his work on pepsin Buchs (1947) has shown that there is present in pepsin an enzyme of the cathepsin type which shows optimal activity at pH 3·5, and our observations suggest that both pepsin and pancreatic extracts contain enzymes of this type, active at pH 3–3·5, and that these are the ones responsible for the antitoxin splitting. Since all the other enzymes tested show a similar optimal pH value for antitoxin splitting it is probable that they all contain an enzyme of the cathepsin type, but this has not yet been definitely established.* The methods used for fractionation of the enzymes and for determination of the proteolytic activity are not given in detail here as it is proposed to make this the subject of a further communication.

The digestion method, using pepsin, is now a well-established one for the purification of antitoxins for clinical use. Since other crude enzymes have been shown to have the property of splitting the antitoxin molecule, it may be worth while pointing out the advantages of using pepsin. Crude pepsin does not appear to contain any enzymes active at pH values more alkaline than pH 5–6, and the enzyme activity is largely destroyed at slightly alkaline pH values. In the antitoxin refining process the enzyme used is almost completely inactivated after it has done the necessary splitting of the antitoxin molecule. This is not true for either crude trypsin or papain; other enzymes are present which are active at neutrality or slightly alkaline pH values, and these are not readily destroyed or removed from the antitoxin. After the antitoxin molecule has been split at pH 3·8, the portion which carried the antitoxin is considerably more susceptible to the action of the enzymes, which act at more alkaline pH values. By the use of either crude trypsin or papain at pH 3·8 we have been able to purify antitoxin to the same degree as with pepsin, but whereas the pepsin-refined antitoxin shows little or no loss of antitoxin units when kept in the cold room for many years, those prepared using trypsin or papain show very rapid deterioration and fall of antitoxic titre.

* Since this paper was written the presence of a large concentration of an enzyme with this pH optimum has been demonstrated in papain.

SUMMARY.

It has been shown that at 37° the apparent optimal pH value for the action of pepsin in splitting the antitoxin molecule is 3·8 ; at lower temperatures the pH value is nearer 3·2.

A similar optimal pH value has been found for all the other enzymes examined. We have extracted from pancreas and partially purified an enzyme with optimal activity in this region. Samples of crystalline trypsin and chymotrypsin were found to have weak activity at pH 3·8, but this is considered to be due to contamination of these crystalline enzymes with traces of another enzyme.

It is suggested that the enzyme responsible for splitting the antitoxin belongs to the class known as " cathepsin enzymes," and is present in all the crude enzymes examined.

A more detailed examination of pepsin has shown that it also contains a group of enzymes with their pH optima at different values ; one of these, of the cathepsin type, appears to be responsible for antitoxin splitting at pH 3·8. By fractionation of pepsin we have obtained material which shows a sharp optimum activity at pH 1·25 and none at pH 3 (haemoglobin substrate). This fraction had no antitoxin splitting activity at pH 3·8.

All the evidence which we have obtained suggests that a special enzyme of the cathepsin type is necessary for splitting the antitoxin molecule into two parts, one with antitoxin activity and the other without activity.

One of us (C. G. P.) wishes to thank Dr. Northrop, Dr. Taylor and Dr. E. Work for samples of crystalline trypsin or chymotrypsin used in this work.

We also wish to thank Dr. C. L. Oakley for the assays on *Cl. welchii* antitoxin and E. L. Fenton and W. A. Jones, who carried out determinations of enzyme activity against haemolglobin.

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THE USE OF *V. CHOLERAE* FILTRATES IN THE DESTRUCTION OF NON-SPECIFIC INHIBITOR IN FERRET SERA.

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SINCE the haemagglutination-inhibition reaction was first employed in the serological diagnosis of influenza (Hirst, 1942) the presence of inhibitors of haemagglutination in normal sera has been recognized as a disadvantage in the use of the test. Three types of inhibitor have been found :

1. McCrea (1946) described an inhibitor in normal rabbit sera which inhibited agglutination by unheated influenza A viruses, was precipitated by 33 per cent saturation with ammonium sulphate along with gamma globulin, and was inactivated by heating at 62° for 30 minutes.

2. Francis (1947) noted that many normal sera inhibited heated influenza viruses to high titre. The so-called Francis inhibitor has been extensively investigated and is thought to be a mucopolysaccharide. It is heat stable and resists 100° for 30 minutes (McCrea, 1948), but is inactivated by the receptor-destroying enzyme (RDE) of *V. cholerae* filtrates and by prolonged incubation with influenza viruses (Anderson, 1948). Francis inhibitor has been recognized by Anderson (1950) in the sera and tissues of a number of different species.

3. Ginsberg and Horsfall (1949) described an inhibitor in human, guinea-pig, mouse and rabbit sera which inhibits haemagglutination and prevents infection by influenza viruses. The inhibitor is inactivated by heating at 56° for 30 minutes, and acts only in the presence of calcium. Chu (1951) has investigated an inhibitor present in normal mouse sera, which is active against unadapted but not against mouse-adapted influenza strains. This inhibitor is also inactivated by heating at 56° for 30 minutes and may be identical with that described by Ginsberg and Horsfall.

Hirst (1948) investigated the inhibitor in normal rabbit serum and his conclusions differ from those of McCrea. He found the inhibitor of agglutination by unheated and heated influenza virus to be heat stable (100° for 15 minutes), and to resemble Francis inhibitor in its properties. Recently, Smith and Westwood (1950) have discussed the problems raised, and suggest that the evidence is not convincing that the activities against unheated and heated influenza virus are represented by qualitatively distinct inhibitors. They favour McCrea's concept of the inhibitor as a protein-mucoprotein complex, and agree that the purified mucoid which he studied may have been broken off from a larger molecule incorporating both activities.

The extensive use of ferret sera in investigations of the antigenic characters of influenza virus strains makes the inhibitory action of normal ferret sera of

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particular interest. Burnet and McCrea (1946) have described an inhibitor in normal ferret sera which resembled specific antibody in its properties, being inactivated by heating at 65° for 30 minutes, and Anderson (1950) mentions the presence of Francis inhibitor in normal ferret sera. In order to overcome non-specific inhibition of haemagglutination by normal ferret sera, Mulder and van der Veen (1948) recommended the use of filtrates of *V. cholerae*. These authors noted that crude *V. cholerae* filtrates inactivated the non-specific inhibitor in normal ferret serum with only slight destruction of specific antibody. In a later paper (Van der Veen and Mulder, 1950) they have specifically recommended the use of crude *V. cholerae* filtrates, and have noted a lack of correspondence between the RDE activity and ability to destroy non-specific inhibitor in these filtrates. Also, Chu, Andrewes and Gledhill (1950) and Appleby and Stuart-Harris (1950) have reported that purified preparations of the receptor-destroying enzyme, RDE, did not inactivate the non-specific inhibitor in normal ferret sera. However, crude *V. cholerae* filtrates contain a number of enzymes which might influence the results of haemagglutination-inhibition tests, e.g., the receptor-destroying enzyme, RDE (Burnet and Stone, 1947), a mucinase (Burnet, 1948) and a trypsin-like enzyme (Stone, 1949), and it seemed desirable, therefore, to investigate the nature of the inhibitory activity in normal ferret serum and of the factors in *V. cholerae* filtrates responsible for its destruction. The present paper reports the results of this investigation. We have used the term "non-specific inhibitor" to describe the inhibitor in normal serum for unheated influenza virus, and "Francis inhibitor" for the inhibitor for heated influenza virus.

MATERIALS AND METHODS.

Virus.—The following strains were used :—

Influenza A : WS, PR8, MEL, Shope 15 strain of Swine influenza, FM1, A/Sweden/3/50, A/Belfast/2/51, A/Madrid/2/51.

Influenza B : Lee, Crawley (England, 1946), Warner (Australia, 1948), B/Budapest/1/49, B/Johannesburg/1/50.

Allantoic fluids were used from embryos inoculated at 10 days and incubated for a further 2 or 3 days at 35°.

Red cells.—Fowl cells were washed thrice in normal saline and made up in a 0·5 per cent suspension (v/v) in normal saline. The concentration of cells was calibrated by a photo-electric densitometer. As far as possible, vaccinia-insensitive, inhibitor-sensitive fowl cells were used and they were not kept for longer than 5 days.

Sera.—Ferret sera were stored at 2° with the addition of 1/10,000 merthiolate. They were heated at 56° for 30 minutes immediately before use.

V. cholerae *filtrates.*—The 4Z strain of *V. cholerae* was obtained through the courtesy of Sir Macfarlane Burnet. The preparation of filtrates was as described by Burnet and Stone (1947).

Titration of non-specific inhibitor.—Serial two-fold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of 0·5 per cent fowl cells added. Finally a third volume of virus diluted in saline to give 4–8 partial agglutinating doses (A.D.) was added and the cells allowed to settle at room temperature (20–25°). Readings were made by the pattern method, the end-point being

taken as partial agglutination. Titres in the tables are expressed as the reciprocal of the initial dilution of serum present at the end-point.

Titration of Francis inhibitor.—Serial two-fold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of heated Lee virus (heated at 56° for 30 minutes) containing 5 A.D. was added; this was allowed to stand at room temperature for 30 minutes. A third volume of red cells was added and readings were made in the same manner as above.

RDE titration.—The titration was carried out after the method of Burnet and Stone (1947). Serial two-fold dilutions (0·25 ml.) of RDE were prepared in calcium acetate buffer, pH 6·0, and an equal volume of fowl cells added. After incubation for one hour at 37° one drop of saturated solution of sodium citrate was added, followed by 0·25 ml. of MEL virus diluted in saline to give 5 A.D. The end-point of the reaction was taken as partial agglutination, and the titre of RDE is expressed as the reciprocal of the initial dilution of RDE at the end-point.

EXPERIMENTAL.

In a study of haemagglutination-inhibition by normal ferret sera there were three variables to consider—serum, red cells and virus. The normal ferret sera which we studied came from an inbred stock of ferrets bred in the National Institute for Medical Research Farm at Mill Hill, and their sera were surprisingly uniform in inhibitor content. Much more variable were the results obtained with red cells from different fowls and with different strains of influenza virus.

Haemagglutination-inhibition titres with different fowl red cells.

Stuart-Harris (1943) drew attention to the wide variation of specific anti-haemagglutinin titres with influenza B viruses when different fowl cells were used. Table I shows the same phenomenon.

TABLE I.—*Haemagglutination-Inhibition Titres with Different Fowl Cells.*

Serum.	Red cells.	
	Fowl 11.	Fowl 12.
Normal ferret serum . . .	120	<10
Immune ferret serum . .	480	20

5 A.D. of B/Johannesburg/1/50 virus were used.

Cells of fowl 12 give very low inhibitory titres in tests with B/Johannesburg/1/50 virus and normal and immune ferret sera. However, fowl 11 and 12 cells gave identical inhibition titres in tests with A/Sweden/3/50 virus. Cells such as those of fowl 12 are described by Anderson, Burnet and Stone (1946) as "inhibitor-insensitive" and they were not used in these experiments. The other fowl cells which we used were all "inhibitor-sensitive," and gave titres not greatly different from those of fowl 11.

It was also important to avoid the use of vaccinia-positive fowl cells, since crude filtrates of *V. cholerae* were found to agglutinate vaccinia-positive fowl cells (*vide infra*).

Haemagglutination-inhibition titres with different virus strains.

Large variations in inhibitory titres were encountered in tests with different virus strains. The highest inhibitory titres, of roughly 5000 when inhibitor-sensitive cells were used, were found in titrations of normal ferret sera with heated influenza B virus (Francis, 1947). Next, a freshly isolated strain of influenza A virus (A/Belfast/2/51) showed a non-specific inhibitory titre of 1280 when first egg passage amniotic or allantoic fluid in the "intermediate phase" (Burnet and Stone, 1945) was used. The same virus after one further allantoic passage was now in the "D phase" (Burnet and Bull, 1943), and was inhibited by normal ferret sera to a titre of 30. The influenza B viruses which we tested generally gave inhibitory titres of 100–400, and a number of A and "A-prime" strains gave inhibitory titres of the order of 20–80. Swine virus was not inhibited by normal ferret serum (cf. Smith and Westwood, 1949). From the strains examined, B/Johannesburg/1/50 and Crawley viruses were selected and used in the majority of experiments. It was found that there was an inverse linear relationship between the amount of virus and the inhibitory titre of serum. When it was necessary to magnify the inhibitory titre of a serum, small amounts of virus, 3–4 partial agglutinating doses, were used.

Destruction of non-specific inhibitor in normal ferret serum by filtrates of V. cholerae.

Mulder and van der Veen's (1948) observation on the destruction of non-specific inhibitor in normal ferret sera by crude filtrates of *V. cholerae* was readily confirmed. As an aid in identifying the active factor in the *V. cholerae* filtrates an investigation of its properties was carried out.

1. *Effect of pH.*—A series of two-fold dilutions (0·2 ml.) of crude *V. cholerae* filtrate was prepared in phosphate buffer and to each dilution was added 0·05 ml. of normal ferret serum. After overnight incubation (16 hours) at 37° the mixtures were inactivated by heating at 56° for one hour and then titrated for inhibitor against Crawley virus. The results are shown in Table II; destruction of non-specific inhibitor was favoured by a slightly acid pH.

TABLE II.—*pH of Action of* V. cholerae *Filtrate on Non-Specific Inhibitor.*

Dilution of *V. cholerae* filtrate.	Inhibitory titre at pH		
	6·3.	7·0.	8·0.
1:2	<10	<10	15
1:4	<10	30	60
1:8	60	60	120
1:16	80	100	140
1:32	120	120	140
∞	120	160	160

3 A.D. of Crawley virus were used.

2. *Heat stability.*—It was found that the active factor in *V. cholerae* filtrates was completely destroyed by heating at 56° for 30 minutes. When the filtrate was diluted in calcium acetate buffer, pH 6·0, and then heated at 56° for 30 minutes, no destruction of activity occurred.

3. *Calcium requirement.*—Dilutions of *V. cholerae* filtrate were prepared in (1) acetate buffer, pH 6·0, (2) acetate buffer plus 0·1 per cent calcium chloride,

and (3) acetate buffer plus 2 per cent sodium citrate. Titrations of the activity of the *V. cholerae* factor in these diluents were then carried out as above. The results are shown in Table III.

TABLE III.—*Effect of Calcium and Sodium Citrate on* V. cholerae *Factor.*

Dilution of *V. cholerae* filtrate.	Diluent for *V. cholerae* filtrate.		
	Acetate.	Acetate plus calcium.	Acetate plus citrate.
1:2	<10	<10	160
1:4	<10	25	160
1:8	15	30	160
1:16	40	40	160
∞	160	160	160

The table shows the non-specific inhibitory titres against 3 A.D. of Crawley virus.

Table III shows that citrate completely inhibited the action of the *V. cholerae* factor, but the addition of 0·1 per cent calcium chloride did not increase the action.

4. *Adsorption to and elution from fowl red cells.*—It is known that the receptor-destroying enzyme of *V. cholerae*, RDE, is adsorbed to fowl red cells and readily elutes following enzymic inactivation of the influenza red cell receptors (Burnet and Stone, 1947). Experiments showed that the factor responsible for destruction of non-specific inhibitor in normal ferret serum was similarly rapidly adsorbed on to fowl cells at 2°, and could be eluted into saline on incubation at 37°. A comparison was then made of the rate of adsorption and elution of the active factor and RDE.

Washed, packed fowl cells in a final concentration of 5 per cent (v/v) were added to filtrates of *V. cholerae* at 2°. After varying periods the cells were centrifuged at 2° and the supernatants removed. Saline was added to the same volume and elution was carried out for varying periods at 37°. The supernatants and eluates were then titrated for (1) ability to destroy non-specific inhibitor in normal ferret serum, (2) RDE activity. It was found that the two activities showed a corresponding drop in titre on absorption with red cells and a corresponding rise in titre on elution. The results of a representative experiment are shown in Table IV.

TABLE IV.—*Effect of Absorption on Red Cells and Elution on* V. cholerae *Factor.*

Material tested.	Non-specific inhibitory titre. *V. cholerae* dilution.						RDE titre.
	1:1.	1:2.	1:4.	1:8.	1:16.	∞	
V. cholerae filtrate	<10	<10	<10	20	40	120	96
Filtrate absorbed with fowl cells	40	40	60	80	120	..	8
Eluate	<10	<10	<10	30	60	..	64

Table shows titration of untreated *V. cholerae* filtrate, filtrate absorbed with fowl cells for 1 min. at 2° and then eluted for 30 min. at 37°. The materials were titrated for their action on non-specific inhibitor in normal ferret serum and for their RDE titre.

5. *Presence of active factor in purified preparations of RDE.*—A purified preparation of RDE was available through the courtesy of Mr. G. L. Ada of the Walter and Eliza Hall Institute, Melbourne. The RDE was supplied in dried form, in ampoules containing 40,000 units each, and the material was made up in 2 ml. of calcium acetate buffer, pH 6·0.

Dilutions of RDE were prepared in acetate buffer, pH 6·0, and were tested for their action on the non-specific inhibitor in normal ferret serum. The results of an experiment are shown in Table V.

TABLE V.—*Action of Purified RDE on Non-Specific Inhibitor in Normal Ferret Serum.*

RDE dilution.	Inhibitory titre.
1:1	120
1:2	30
1:4	<10
∞	80

8 A.D. of Crawley virus were used.

The undiluted, purified RDE had no action on non-specific inhibitor, but the same material was fully active when diluted 1 in 4. This inhibitory zone has not been investigated fully, owing to difficulty in reproducing the effect. Thus, in experiments with other batches of purified RDE, inhibitor destruction occurred with undiluted and diluted RDE (Table VII).

Calcium requirement of inhibitor-destroying factor of V. cholerae.

These results show that the factor in *V. cholerae* filtrates which destroys non-specific inhibitor in normal ferret sera shows a striking resemblance in properties to the receptor-destroying enzyme, RDE (Burnet and Stone, 1947). The only apparent difference encountered was in calcium requirement. Both reactions were inhibited by 2 per cent sodium citrate, but the action of RDE was increased by the addition of 0·1 per cent calcium chloride, whereas the action of the inhibitor-destroying factor was, if anything, slightly diminished. However, the difference would be no more than an apparent one if the serum supplied the calcium necessary for the reaction. This was tested by estimating the RDE titre of a *V. cholerae* eluate in the following diluents:

1. Acetate buffer, pH 6·0.
2. Acetate buffer plus 0·1 per cent calcium chloride.
3. Acetate buffer plus 0·1 per cent calcium chloride plus 2 per cent sodium citrate.
4. Acetate buffer plus normal ferret serum 1/5.
5. Acetate buffer plus normal ferret serum 1/5 plus 2 per cent sodium citrate.

However, it was first necessary to remove non-specific inhibitor from the normal ferret serum; this was carried out by previous overnight incubation of the serum with *V. cholerae* eluate at 37°, and the RDE was then destroyed by heating at 56° for one hour. The results of the RDE titrations are shown in Table VI.

TABLE VI.—*Receptor-destroying (RDE) Titre of a Red Cell Eluate of V. cholerae in Various Diluents.*

Diluent.	RDE titre.
Acetate pH 6·0	2
Acetate+0·1 per cent $CaCl_2$	80
Acetate+0·1 per cent $CaCl_2$+2 per cent sodium citrate	16
Acetate+1/5 inactivated serum	80
Acetate+1/5 inactivated serum+2 per cent sodium citrate	10

It can be seen that the titre of RDE is greatly increased in the presence of either 0·1 per cent calcium chloride or 1/5 normal ferret serum. Citrate has an inhibitory effect on both reagents, suggesting that the effect of the serum is due to its calcium content.

Properties of the inhibitor in normal ferret serum.

These results suggested that the active factor in *V. cholerae* filtrates was RDE, and it might be expected, therefore, that the inhibitory property of normal ferret sera was due to Francis inhibitor. One method of investigating this was as follows:

A series of dilutions of purified RDE in acetate buffer was prepared, and to each was added a constant amount of normal ferret serum. After overnight incubation at 37° the sera were heated for one hour at 56°, and each dilution was titrated for (*a*) non-specific inhibitor against Crawley virus and (*b*) Francis inhibitor against heated Lee. The results are shown in Table VII, where it is seen that the highest dilution of RDE which destroys the inhibitors is in each case 1/128. This suggests that the Francis inhibitor is responsible for both the high titre inhibition of heated virus and the lower titre inhibition of unheated virus.

TABLE VII.—*Action of Purified RDE on Non-specific Serum Inhibitor and Francis Inhibitor.*

Titration.	RDE dilution.										
	1:1.	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	∞
Non-specific inhibitor	<10	<10	<10	<10	<10	<10	<10	<10	15	60	80
Francis inhibitor	<50	<50	<50	<50	<50	<50	<50	<50	100	1400	4800

Non-specific inhibitor was titrated against 6 A.D. of Crawley virus. Francis inhibitor was titrated against 6 A.D. of heated Lee.

Heat stability of inhibitor in normal ferret serum.—Normal ferret serum was diluted 1 in 10 in normal saline, and aliquots were heated at 65° and 56° for 30 minutes, and then titrated for non-specific inhibitor and Francis inhibitor. The results of such an experiment are shown in Table VIII.

A two-fold reduction in both inhibitory titres occurred on heating at 65°. Altogether 13 normal sera from our ferret stock have been tested, and in all cases

TABLE VIII.—*Heat Stability of Inhibitors in Normal Ferret Serum.*

Titration.	Serum heated 30 minutes at—	
	56°.	65°.
Non-specific inhibitor . . .	640	320
Francis inhibitor . . .	6400	3200

Non-specific inhibitor was titrated against 3 A.D. of Crawley virus. Francis inhibitor was titrated against 6 A.D. of heated Lee.

the non-specific inhibitory titre was unaffected or only slightly reduced following heating at 65° for 30 minutes. In addition, a batch of pooled normal ferret serum obtained through the courtesy of Dr. P. von Magnus of the State Serum Institute, Copenhagen, and two normal sera obtained from bought ferrets through the courtesy of Professor C. H. Stuart-Harris, were examined, and the non-specific inhibitor found to be resistant to heating at 65° for 30 minutes. The results were the same whether the non-specific inhibitor was tested against A or B strains or against a newly isolated A strain in the first egg passage.

Action of influenza virus on non-specific inhibitor in normal ferret serum.

Incubation of normal ferret serum with influenza virus (strains A/Madrid/2/51 and Lee), followed by heating at 65° for 30 minutes to destroy all remaining haemagglutinin, produced a drop in non-specific inhibitory titre and a corresponding drop in Francis inhibitor, although complete destruction of these inhibitors did not occur. The results of an experiment of this kind are shown in Table IX.

TABLE IX.—*Effect of Influenza Virus on Non-specific Serum Inhibitor and Francis Inhibitor.*

Serum incubated with—	Titration.	
	Non-specific inhibitor.	Francis inhibitor.
A virus	10	75
B virus	30	100
Saline control . . .	60	300

Serum was incubated overnight at 37° with 100 A.D. of A/Madrid/2/51 and 50 A.D. of Lee. Inactivation—30 minutes at 65°. Non-specific inhibitor was titrated against 5 A.D. of Crawley virus. Francis inhibitor was titrated against 4 A.D. of heated Lee.

Action of trypsin on non-specific inhibitor in normal ferret serum.

A preparation of crystalline trypsin was kindly supplied by Dr. W. J. Elford. When diluted in phosphate buffer, pH 8·0, in a concentration of 0·1 per cent and incubated for 2 hours at 37° with normal ferret serum it was found to destroy both the non-specific inhibitory titre and the Francis inhibitor (Burnet, McCrea and Anderson, 1947).

Use of crude V. cholerae *filtrates in destroying non-specific serum inhibitor.*

The use of crude *V. cholerae* filtrates for the destruction of non-specific serum inhibitor is undesirable, since they may contain a number of complicating factors.

The following factors, which are known to occur in crude *V. cholerae* filtrates, may interfere with the results of haemagglutination-inhibition tests.

1. A proteinase is present which may be identical with the trypsin-like agent described by Stone (1949). The proteinase destroys specific antibody. Most workers who have used crude *V. cholerae* filtrates have noted destruction of specific antibody, and we have repeatedly noticed this in our experiments. It can be shown that crude *V. cholerae* filtrates produce liquefaction of gelatin. When RDE was absorbed by fowl red cells all detectable proteinase activity remained in the supernatant fluid; eluates did not produce liquefaction of gelatin.

2. One *V. cholerae* filtrate when incubated with normal ferret serum for short periods (1 hour) at 37°, followed by heating for one hour at 56°, has shown the property of increasing the inhibitory titre ten-fold. The responsible factor is not inactivated by heating at 60° for 30 minutes, but is destroyed by boiling for 5 minutes.

3. Crude *V. cholerae* filtrates contain a lipoid which agglutinates vaccinia-sensitive cells (Stone, 1946) to a titre of approximately 32. This was previously observed by Chu (1948). It was also found that a lipoid haemagglutinin was present to a titre of 640 in a preparation of purified RDE supplied by Mr. G. L. Ada. Our results do not suggest that the lipoid and RDE are intimately associated; thus the lipoid agglutinin was absorbed from *V. cholerae* filtrates by vaccinia-sensitive and insensitive fowl cells (*cf.* Burnet and Boake, 1946) but did not elute along with the RDE.

Use of V. cholerae *red cell eluates in destroying non-specific inhibitor.*

It seems clear from the results that the non-specific inhibitor in normal ferret serum corresponds to the Francis inhibitor and that it can be inactivated by RDE. Red cell eluates of RDE are easily prepared, and afford a relatively simple method of obtaining the enzyme free from significant amounts of proteinase. Eluates have been prepared by absorption with 5 per cent fowl cells for 1 minute at 2°, followed by elution into normal saline for 30 minutes at 37°; the eluates were then diluted in acetate buffer pH 6·0 and titrated for their ability to destroy non-specific inhibitor. They have been used at the highest effective dilution. A number of experiments have been carried out with RDE eluates in studies of the antigenic structure of recently isolated strains of influenza A and B viruses; sera treated in this way have shown complete destruction of non-specific inhibitor with no reduction in the titre of specific antibody.

DISCUSSION.

The conclusions which these results suggest are that the active factor in *V. cholerae* filtrates is the receptor-destroying enzyme, RDE, and that the non-specific inhibitor in the ferret sera under investigation is largely, if not wholly, Francis inhibitor. The evidence for this can be summarized briefly:

1. A slightly acid pH (6·0) favours the action of the active factor and of RDE.

2. The active factor and RDE are both inactivated by heating at 56° for 30 minutes; both are protected from the destructive effect of heat by diluting in calcium acetate buffer, pH 6·0.

3. Both factors require calcium for their action and both are inhibited by sodium citrate.

4. Both factors are adsorbed to and can be eluted from fowl red cells; absorption and elution of the active factor and RDE occur at the same rate.

5. Purified preparations of RDE destroy non-specific inhibitor; the effective dilution is commensurate with the RDE titre of the preparation.

6. RDE inactivates the non-specific inhibitor and the Francis inhibitor in normal ferret serum at the same rate.

7. The non-specific inhibitor in normal ferret serum is not inactivated by heating at 65° for 30 minutes.

8. The non-specific inhibitor is inactivated on incubation with influenza virus.

The difference between the present results on the activity of purified preparations of RDE and those of previous workers may be due to the pro-zone effect described above; some purified preparations of RDE were found to be ineffective in destroying non-specific inhibitor when tested undiluted but were active when diluted. The ferret sera which we have examined have not contained significant amounts of the heat labile (65°) globulin inhibitor described by Burnet and McCrea (1946). It may be that sera from some ferret stocks differ in their inhibitor content from those which we have examined. In general, our results with ferret sera are in agreement with the conclusions reached by Smith and Westwood (1950) in their study of the inhibitor in normal rabbit sera.

The use of red cell eluates of *V. cholerae* is clearly preferable to that of the crude filtrates. By this means it may be possible to obtain a more accurate picture of the antigenic structure of influenza viruses, uncomplicated by non-specific inhibitory effects. How important this is can be seen from present studies of influenza B viruses (Bozzo, in preparation), in which the titre of non-specific inhibitor in an immune ferret serum may be greater than the titre of specific antibody for a particular virus under test. The use of RDE-treated ferret sera has been invaluable in the study of the large number of A-prime strains received at the World Influenza Centre following the recent epidemic in Europe. It has been possible to use antisera in the confident knowledge that treatment with RDE eluate destroys all non-specific inhibitor without in any way affecting the titre of specific antibody. It may be worth while emphasizing, however, that this method will not be applicable to sera from other species unless it is found that their non-specific inhibitory titre is due predominantly to Francis inhibitor.

SUMMARY.

The factor in *V. cholerae* filtrates responsible for the destruction of non-specific inhibitor in normal ferret serum has been identified as the receptor-destroying enzyme, RDE.

The non-specific inhibitor in 16 normal ferret sera has been found to be due to the activity of Francis inhibitor.

The use of *V. cholerae* eluates is recommended for the destruction of non-specific inhibitor in ferret sera.

We should like to thank Mr. E. Owen for his most valuable technical assistance.

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RESPIRATION AND CELL DIVISION IN THE RED BLOOD CELLS OF THE CHICKEN EMBRYO.

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In the normal development of the midbrain of the chicken embryo it has been shown that there is a linear relationship between the number of dividing cells and the rate of aerobic glycolysis (O'Connor, 1950*a*). A dependence of cell division on aerobic glycolysis was thus suggested and further supported by investigations which demonstrated mitotic damage when aerobic glycolysis was inhibited by fluoride and iodoacetate. The concentrations of both substances producing this effect did not inhibit respiration (O'Connor, 1950*b*).

In order to investigate the possibility of similar relationships in the circulating blood cells of the chicken embryo the variations of cell division, aerobic glycolysis, respiration and respiratory quotient have been measured during normal development.

MATERIALS AND METHODS.

Preparation of blood for examination.

Chicken embryos of the third to ninth days of incubation were used, blood being removed from the heart or from a blood vessel by a capillary pipette. Except where a modification is described the blood was discharged into the following medium, anhydrous compounds being used: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., glucose 0·1 g., water 100.0 ml., to which 10 ml. of M/15 phosphate buffer (Sørensen) was added to produce pH 7·4.

The cells were washed and concentrated to produce a suspension containing 10^5–10^6 cells per c.mm. No attempt was made to separate red cells from white, since the latter were too few to affect measurements made on all the cells. This investigation is therefore to be considered as one on the circulating red blood cells.

The measurement of cell volume.

Since the size of the circulating red cells decreases with development, the measurements of respiration and aerobic glycolysis were related to a unit volume of cells rather than to a unit number. The average cell volume was determined from the haematocrit, portions of the cell suspensions being centrifuged in capillary tubes of constant internal diameter. Since the cells were suspended in the medium described it was possible that some difference in the tonicity of cells and medium might affect the haematocrit reading. However, microscopic measurements revealed no significant differences between the average diameter

of cells suspended in the medium and of equivalent cells suspended in embryonic plasma directly after removal from the embryo.

The measurement of cell division.

A small drop of the suspension was placed on a microscope slide and mixed with the following stain: Lacmoid (resorcin blue), 2·2 g.; glacial acetic acid, 100·0 ml. This solution was diluted with an equal volume of water and filtered immediately before use. The mixture of suspension and stain was covered with a cover-slip, ringed with vaseline and examined forthwith. An even distribution of dividing cells was obtained and the percentage determined in 2000–3000 cells.

The measurement of the rate of respiration and respiratory quotient.

The Cartesian diver micromanometer, as previously described (O'Connor, 1950*a*), was used. About 10 c.mm. of the cell suspension, containing between 1 and 5 million red cells, was placed in the divers and rates of respiration determined per million cells. By means of the cell volume the rate of respiration was expressed as c.mm. oxygen per c.mm. red cells per hour. The measurements were not affected by variations in the amount of haemoglobin bound to the red cells because the observations were made in an atmosphere of oxygen and the haemoglobin remained saturated throughout.

The measurement of the rate of aerobic glycolysis.

This was determined as described previously (O'Connor, 1950*a*). The cell suspension was made in a bicarbonate-containing medium and the measurements made in Cartesian divers containing an atmosphere of 95 per cent oxygen/5 per cent carbon dioxide.

RESULTS.

Table I summarizes the results obtained from embryos of 3 to 9 days' incubation, each figure being obtained from 10–20 separate measurements. It will be seen that throughout the respiratory quotient does not differ significantly from 1·0 and there is no aerobic glycolysis. During development, however, there is a fall in the number of dividing cells and the rate of respiration.

The daily averages for cell division and respiration are plotted in Fig. 1. After the ninth day the rate of respiration continues to fall and these variations are recorded in the figure. In obtaining these daily averages the corresponding

TABLE I.—*Cell Volume, Respiration, Respiratory Quotient, Aerobic Glycolysis and Cell Division in the Circulating Red Cells of the Chicken Embryo.*

	Day.					
	3.	4.	5.	6.	7.	8.	9.
Respiration, c.mm. $O_2/10^6$ cells/hr.	0·47	0·26	0·19	0·14	0·11	0·056	0·044
Cell volume (u^3)	780	570	500	430	380	240	210
Respiration, c.mm. O_2/c.mm. cells/hr.	0·60	0·46	0·38	0·32	0·29	0·24	0·21
Dividing cells (per cent)	4·6	3·6	2·7	1·3	0·2	0	0
Respiratory quotient	1·04	0·98	1·02	0·96	0·98	1·04	1·06
Aerobic glycolysis	0	0	0	0	0	0	0

measurements were not necessarily made on the same blood suspension, and on the third and fourth days of incubation not only are the measurements changing rapidly but, in addition, there is considerable relative variation in the stage of development reached at any given time. For these reasons Fig. 1 includes observations in which the measurements have been made on the same blood sus-

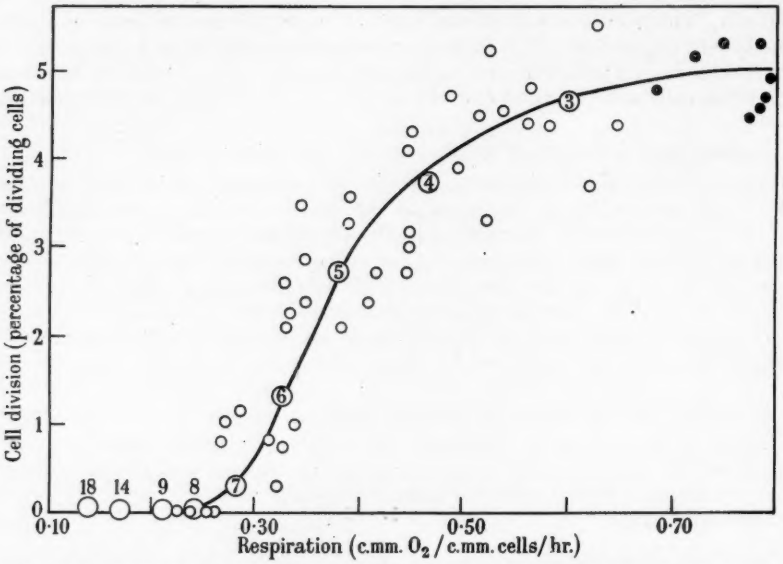

FIG. 1.—The relationship of respiration and cell division in the circulating red blood cells of the chicken embryo. Large circles—daily averages with day indicated. Small circles—corresponding values obtained from the same red cell suspension, the closed small circles being derived from embryos soon after the establishment of the intra-embryonic circulation.

pension ; these are therefore strictly comparable. These measurements include those made on the earliest stages technically possible, that is, on embryos of about 25 somites, soon after the establishment of the intra-embryonic circulation. For this purpose the blood from 5–6 embryos was pooled. These measurements are specifically indicated in Fig. 1.

DISCUSSION.

In considering a possible relationship between the number of dividing cells and the rate of respiration there is the question whether the circulating red cells are homogeneous as regards stage of maturation, and whether cell division is confined to a limited number of the total cells, which are at an earlier stage of development than the remainder. This is unlikely, for in Leishman-stained films no staining differences can be found between the cytoplasm of dividing cells and the immediately adjacent non-dividing cells. Further, the cytological investigations of Dawson (1936) show that throughout incubation the circulating

red cells of the chicken embryo are predominantly of one type, which increases in maturation as incubation proceeds.

Dawson gives figures for the percentage of dividing cells at each day of incubation. In general his figures are somewhat lower than those recorded in Table I, and, in one respect, require comment. On the third day Dawson's figures indicate a rise in the dividing cells from 1·49 per cent to 3·44 per cent, this rise continuing into the fourth day when a decrease, comparable to that recorded here, sets in. It has not been possible to confirm this rise in the percentage of dividing cells on the third day. Blood from three-day embryos at the earliest stage where there was an intra-embryonic circulation gave an average value of 4·8 per cent dividing cells, which is no lower than the average for all three-day embryos. It may be pointed out that Dawson's figures were obtained from the examination of dried smears, while the present results are derived from the wet preparations described. Dry smears were not used because they appeared to give an uneven distribution of dividing cells and because, even in the best preparations, the débris of dividing cells was often seen.

In Fig. 1 the plotted points group themselves around the line drawn, but its significance must be regarded as doubtful owing to the scatter of the individual results. Further, it has to be pointed out that the observations only begin at the third day of incubation when the red blood cells, in terms of haemoglobin formation, have undergone a considerable degree of differentiation and contain 30–40 per cent of the haemoglobin they will eventually acquire.* The observations, therefore, do not cover the earliest stages of red cell development. However, the results obtained on the earliest embryos examined suggest that, to cover the whole range of the variations in respiration and cell division a curved line would best fit the relationship between them.

Although the precise relationship of cell division to respiration cannot be defined there is a difference between these findings and those in the development of the midbrain of the embryonic chicken. There, dividing cells decreased as aerobic glycolysis decreased, while respiration remained constant. In the red cells there is an association between decreasing cell division and decreasing respiration while aerobic glycolysis does not occur. On the other hand, since in both tissues the respiratory quotient is unity, it is probable that the rate of respiration is a measure of the rate of the complete oxidation of carbohydrate, so that in both tissues a decreasing number of dividing cells is associated with a decreasing rate of total carbohydrate metabolism, the two tissues differing in their relationships to the respiratory and glycolytic paths. Further, both tissues can be said to differ from the epidermis of the mouse, where the findings of Bullough (1950) indicate that cell division is dependent on both paths of carbohydrate metabolism.

A possible explanation of these differences may lie in the connection between cell division and cell differentiation. In embryonic tissues, and in tissues capable of regeneration such as skin, the fate of dividing cells is to differentiate, that is, to undergo metabolic processes producing substances characteristic of the tissues concerned. Many observations (e.g., Doljanski, 1930*a*, *b*) show that there is an inverse relationship between cell division and cell differentiation, indicating a linkage between the two processes. Thus, in different tissues, cell division may

* This statement is based on quantitative estimations of haemoglobin content, subsequently to be dealt with in detail.

be linked to different anabolic processes, and this may be the basis for differences in the relationships to the catabolism of carbohydrate. In malignant tissue there is a breakdown of the linkage between cell division and cell differentiation, and it is possible that this breakdown is associated with an alteration of metabolic relationships of cell division, which might explain the differences in the response of normal and malignant or pre-malignant tissues to anti-mitotic agents. Lasnitzki (1948) has shown that urethane inhibits mitosis in cultures of normal cells, but stimulates the growth of C57 sarcoma and adenocarcinoma 63. More recently Green and Savigear (1951) have shown that the inhibitory effect of cortisone and shock on cell division in the skin of the mouse disappears after the application of a carcinogen.

SUMMARY.

In the circulating red blood cells of the chicken embryo the rate of respiration and the number of dividing cells decrease during normal development.

At all stages investigated the respiratory quotient is unity and aerobic glycolysis cannot be demonstrated.

The possibility is discussed that there is in the red blood cells of the chicken embryo a relationship between carbohydrate metabolism and cell division. Comparisons are made with the metabolic relationships of cell division in other tissues.

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A COMPLEMENT-FIXING ANTIGEN OF *BACT. COLI* BACTERIOPHAGE T5: ITS BEHAVIOUR DURING VIRUS GROWTH.

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COMPLEMENT-FIXING antigens of animal viruses have long been known, and complement-fixation reactions have been used both in the diagnosis of virus infections and in studies of the antigenic relationships of different viruses. A successful example of the application of the reaction to the study of virus multiplication is the work of Hoyle (1945, 1948) on the influenza virus. Having established the presence in human convalescent sera of complement-fixing antibodies to infective virus and to a "soluble" antigen obtained from infected mouse lung or allantoic fluid, Hoyle proceeded to study the occurrence of this "soluble" antigen in infected cells of the chorio-allantois of the chick embryo. His findings indicated that, during the growth cycle in the chick, the appearance of the "soluble" antigen preceded that of the infective particles.

A search of the literature has revealed no reports of the use of the complement-fixation reaction with bacterial viruses, all reported serological studies being concerned with neutralization of virus infectivity or with agglutination of bacteria coated with phage. It may be that attempts to find sera containing complement-fixing antibodies have been unsuccessful and have therefore not been described.

This paper reports the occurrence in a rabbit serum of complement-fixing antibody for coli phage T5, and the use of this antibody to study the growth cycle of the phage in cells of *Bacterium coli*, strain B.

MATERIAL AND METHODS.

A set of the coli phages of the T series, T1–T7, was obtained from Professor J. L. Still, to whom they had been sent by Dr. M. Delbrück. The strain of *Bact. coli* also came from Professor Still.

Stock phages prepared in nutrient veal broth containing 0·15 per cent glucose and filtered through gradocol membranes had a titre of 10^9–10^{11}/ml. It was necessary to add $CaCl_2$ to a concentration of 0·001 M to ensure efficient growth of T5 in this medium. Phage titrations were made on glucose veal agar plates by the surface plating method, 0·01 ml. inocula being used and each plate accommodating 4 inocula. Plaque counts were made after incubation overnight at 37°.

Counts of viable coli cells were made by the method of Miles and Misra (1938).

Phage neutralization tests were made by the method of Burnet, Keogh and Lush (1938).

All dilutions of phage and cells made for counting were carried out in broth held in an ice-water bath.

In the complement-fixation tests, 0·1 ml. of each of the reactants was used. After fixation for 1 hour at 37° the haemolytic system was added. This consisted of sheep's cells sensitized with 2½ MHD of rabbit haemolysin, 0·2 ml. of cells being added to give a final concentration of 1 per cent cells and a final volume of each tube of 0·5 ml.

EXPERIMENTAL.

Complement Fixation with Rabbit Serum and T5 Phage.

Preparation of the serum.

Three rabbits were injected intravenously with broth filtrates of phages T4, T5 and T7 respectively. The course of injections was 0·5, 1·0, 1·5 and 2·0 ml. at 3-day intervals. After a further 7 days a trial bleed was made, and the serum examined for phage neutralization and for complement-fixing antibody. The sera from the rabbits immunized with T4 and T7 contained high titres of neutralizing antibody (1 in 20,000) but gave no complement fixation with their homologous phages. The serum from the rabbit immunized with T5 contained a comparatively small amount of phage-neutralizing antibody, but it also showed complement fixation with T5. This rabbit was bled out and the properties of the serum studied in detail.

Phage neutralization with anti-T5 serum.

Phage neutralization tests set up with T5 and the serum showed that the end-point of the serum (i.e., that dilution giving neutralization of 80 per cent. of the phage infectivity) was 1/120. The neutralization curve was the typical S-shaped curve obtained by Burnet, Keogh and Lush (1937) with other phage-serum systems. There was no evidence of a flattened neutralization curve such as they found with sera obtained early in the course of immunization.

Complement fixation with anti-T5 serum.

The general behaviour of the antiserum-phage system was studied by the "chessboard" technique of Hoyle (1945). The results of a typical titration using falling dilutions of antigen and antibody and 2 MHD of complement and fixing for 1 hour at 37° are given in Table I. Taking 50 per cent haemolysis as

TABLE I.—*Titration of Complement Fixation with T5 Homologous Anti-serum and 2 MHD Complement.*

Phage dilution.	Serum dilution. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
Undil.	0	0	0	0	0	0	0	+
1/2	0	0	0	0	0	0	0	+
1/4	0	0	0	0	0	0	+	++
1/8	0	0	0	0	0	0	++	++
1/16	0	0	0	0	±	±	++	++
1/32	0	0	0	0	++	++	++	++
1/64	+	++	++	++	++	++	++	++
1/128	++	++	++					

T5 = Broth filtrate containing $5·9 \times 10^{10}$ infective particles/ml.
0 = No haemolysis.
± = Trace of haemolysis.
+ = 50 per cent haemolysis.
++ = Complete haemolysis.
Fixation 1 hour at 37°

the end-point, the maximal antibody titre was 1/256. The phage filtrate used in this titration contained $5{\cdot}9 \times 10^{10}$ infective phage particles/ml. and gave a maximal antigen titre of 1/64.

Further "chessboard" titrations were made with the same phage filtrate and 4 MHD and 6 MHD of complement. The results given graphically in Fig. 1, and using the 50 per cent end-point show the reduction in titres with increasing amounts of complement. There are no indications of zone phenomena or of an optimal antigen titre such as were found by Hoyle with influenza antigens. The shape of the curves corresponds to that stated by Hoyle to occur commonly when the particle size of the antigen is large.

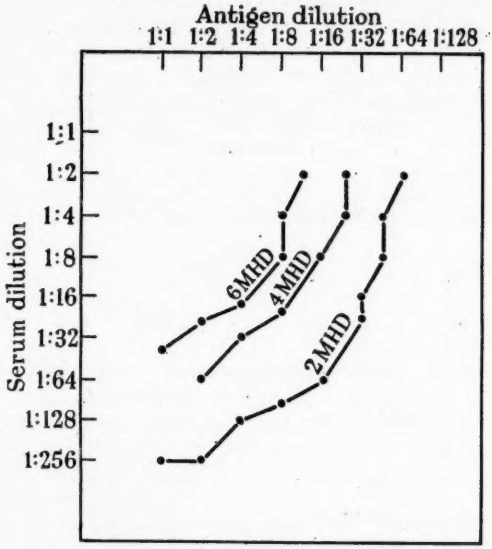

FIG. 1.—Titration of complement fixation with phage T5 and antiserum. Falling dilutions of antigen and antibody, using 2, 4 and 6 MHD of complement. End-point = 50 per cent haemolysis.

In Fig. 2 the maximal antigen titres have been plotted against the maximal antibody titres obtained with increasing amounts of complement. In Fig. 3 the maximal antigen and the maximal antibody titres have been plotted against the various doses of complement. In both figures the straight lines indicate that the ratios of maximal antigen to maximal antibody are independent of the dose of complement present, but that the actual titres obtained do depend on the amount of complement present.

Inspection of the curves in Fig. 1 showed that, with 2 MHD of complement a 1 in 8 dilution of serum should give nearly as much fixation as a 1 in 2 dilution. In order to conserve the stocks of serum, a standard dose of 2 MHD of complement and a 1 in 8 dilution of serum were used in subsequent titrations of antigen.

Some increase in the sensitivity of the reaction could be demonstrated when fixation was carried out for 4 hours in the refrigerator, but since adequate fixation

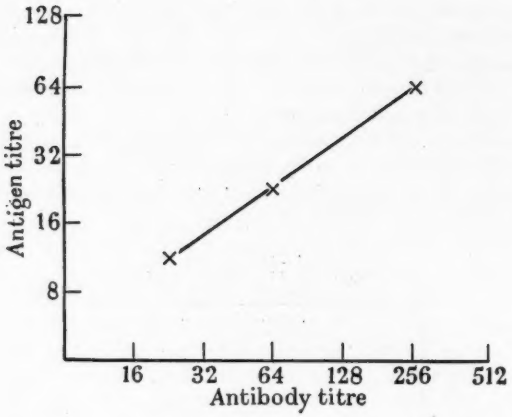

FIG. 2.—Complement fixation with phage T5 and antiserum. Maximal antigen titres plotted against maximal antibody titres with 2, 4 and 6 MHD complement. Data derived from Fig. 1.

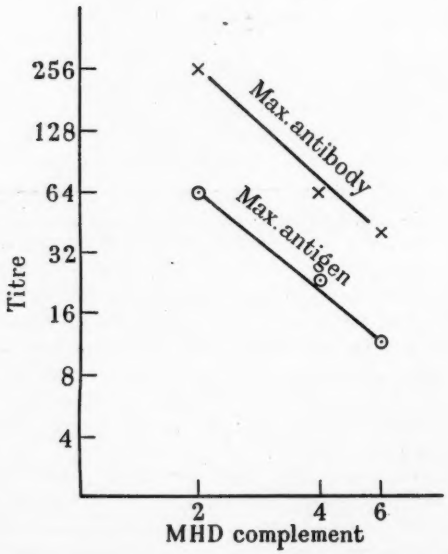

FIG. 3.—Complement fixation with phage T5 and antiserum. Maximal antigen and maximal antibody titres obtained with 2, 4 and 6 MHD complement. Data derived from Fig. 1.

took place at 37° this increase was not considered sufficient to warrant use of the longer period of fixation.

Relationship of antigen titre to titre of infective phage.

The "chessboard" titrations having established the presence in phage filtrates of an antigen capable of reacting in the complement-fixation reaction, it was necessary to determine whether the titre of this antigen would be related directly to the titre of infective phage particles present in the filtrates and whether the reaction was specific for T5.

A freshly prepared phage preparation was filtered through a gradocol membrane stated to be of 130 mμ. APD. The titres of infective phage in the unfiltered and the filtered material were determined, and complement-fixation tests were set up using undiluted phage and falling dilutions of antibody. The results, in Table II, show that a 70 per cent reduction in infective phage was accompanied by a comparable reduction in the end-point of the antibody titre of the serum, which was reduced from 1 in 64 to 1 in 24 (63 per cent).

TABLE II.—*Relationship between Titre of Infective Phage and Antibody Titre.*

Phage.	Infective titre/ml.	Per cent. reduction.	Antibody titre.	Per cent. reduction.
Filtered through 800 mμ. APD	$1{\cdot}6 \times 10^{10}$	..	1/64	..
Filtered through 130 mμ. APD	$4{\cdot}8 \times 10^{9}$	70	1/24	63

Numerous other titrations of antigen titre made during the course of subsequent experiments showed that the antigen titre could be related linearly to the titre of infective phage, provided that freshly prepared filtrates in which no inactivation of infectivity had occurred were used. Calculations also showed that the minimum amount of antigen necessary for fixation was the equivalent of 1×10^9 infective particles/ml.

Phage filtrates whose infectivity was destroyed by heating for 10 minutes at 73° were still as active antigenically as the uninactivated filtrates. Furthermore, the antigen could be precipitated from the broth lysate with 4 volumes of acidified absolute alcohol in the cold. Re-suspension of the precipitate in distilled water resulted in a clear solution which contained no infective phage, but which gave complement fixation to the same titre as the original material.

It was therefore concluded that the complement-fixing activity of phage preparations, although related linearly to the number of infective particles originally present in the preparation, did not depend on the presence of actively infective phage. The phage antigen acted equally well whether associated with infectivity or not. Lysates in which inactivation of phage particles had occurred would give a higher antigen titre than would have been expected on the basis of their present content of infective virus.

Specificity of the reaction.

Since the broth preparations of phage used for immunization and in the complement-fixation tests might be expected to contain some antigenic material derived from the lysed *Bact. coli* cells, it was necessary to determine whether any

of the activity of the serum was due to coli antibody. A volume of 1 ml. of serum was absorbed with the growth from 5 agar slopes of *Bact. coli* suspended in 2 ml. of saline. After centrifugation, complement-fixation tests were set up with the absorbed serum and phage filtrates. No reduction in the antibody titre of the serum or in the antigen titre could be found. However, the serum was anti-complementary up to a dilution of 1 in 12, and further tests showed that this anti-complementary action was due to the bacterial cells or to material extracted from them by the saline. It was therefore impossible to test non-absorbed serum for antibody to bacterial cells, and it cannot be stated with certainty that the serum did not contain a small amount of antibody to *Bact. coli*. Some of the results to be described later could, in fact, have been due to such an antibody. However, when large amounts of phage antigen were present in the material being tested, the presence of this antibody to *Bact. coli* did not interfere with the phage antigen titres.

The cross-reactions of the serum with the other phages of the T series were also examined, both for virus neutralization and complement fixation. A summary of the results is given in Table III. There were no cross-reactions in the

TABLE III.—*Reactions of Anti-T5 Serum with Phages of T Series.*

Phage.	Infective particles/ml.	Neutralizing antibody titre.	Complement-fixation titres.	
			Max. antigen.	Max. antibody.
T5	$5{\cdot}9 \times 10^{10}$	120	64	256
T1	$3{\cdot}7 \times 10^{10}$	*nil*	4	4
T2	$2{\cdot}9 \times 10^{10}$	not done	1	2
T3	4×10^{10}	*nil*	6	24
T4	$1{\cdot}7 \times 10^{10}$	1/2	2	6
T6	$4{\cdot}5 \times 10^{10}$	not done	2	8
T7	$2{\cdot}6 \times 10^{10}$	*nil*	4	16

virus neutralization tests with the exception of some activity with T4 at a serum dilution of 1 in 2. This is in accord with the results of Delbrück (1946). In the complement-fixation tests the maximal antigen titres were of a low order, the maximal titre being 1 in 6 with T3. The maximal antibody titres, particularly with T3 and T7, were somewhat higher, and it must be considered whether these cross-reactions may be due to bacterial antigen-antibody reactions rather than to the phage. The fact that serum absorbed with *Bact. coli* cells was anti-complementary at a dilution of 1 in 12 precluded, with one exception, the use of absorbed serum to elucidate this question. In the case of T3, which gave a maximal serum titre of 1 in 24 with unabsorbed serum, repetition of the fixation test with absorbed serum showed a reduction in antibody titre to 1 in 12, the point at which the absorbed serum was anticomplementary. It is reasonable to assume therefore that, in the case of T3 at least, the activity of the serum is due to antibody to *Bact. coli* rather than to the possession by the heterologous phages of an antigen in common with T5. Further support to this assumption is given by the fact that those phages which showed maximal crossing with the T5 anti-serum, namely T3 and T7, are those producing on agar large plaques with marked haloes suggesting the production of a bacterial lysin as well as infective particles. Lysates of these phages may therefore contain larger amounts of bacterial debris than do those produced by the small-plaque phages.

To summarize : rabbit serum against phage T5 contained a complement-fixing antibody specific for an antigen of T5. The antigenic activity of phage preparations could be related to their content of phage particles as measured by the plaque count, but was not dependent on the possession of infectivity by these particles. The serum probably also contained a small quantity of antibacterial antibody which was responsible for crossing with heterologous phages of the T series.

The Behaviour of the Phage Antigen during Phage Multiplication.

The complement-fixation reaction was used to study the behaviour of the phage antigen in infected cells during the latent period. The main requirement for this study was a method whereby infective phage and/or phage antigen could be obtained from the cells during the latent period in quantities sufficient to be detected serologically. Antigen equivalent to a minimal concentration of 1×10^9 phage particles/ml. was necessary for complement fixation. As there was a limiting concentration of viable *Bact. coli* cells in broth cultures, cells grown to a concentration of 10^9/ml. having passed optimal metabolic conditions for phage synthesis, it was necessary to concentrate the infected cells to obtain adequate amounts of antigen. A method whereby the phage could be released from the infected cells before the normal end of the latent period was also required.

The first attempts employing centrifugation of infected cells and grinding with alumina were found to be impracticable, and in addition gave only small yields of antigen. The method finally adopted involved concentration of infected cells by centrifugation and their lysis by the cyanide technique of Doermann (1948, 1949). Doermann has shown that the addition of cyanide to *Bact. coli* cultures infected with T2 or T4 immediately interrupts phage growth, and results in the release of any newly formed infective phage that may be present in the cells. Preliminary experiments showed that this technique could be applied to cells infected with T5. The technique finally employed was as follows :

A 100 ml. quantity of broth was inoculated with 2 ml. of an overnight broth culture of *Bact. coli* B, and grown in a water-bath at 37° with aeration, to a concentration of approximately 5×10^8 cells/ml. Just before addition of the phage a sample was taken for a viable cell count, and 1 ml. of 0·1 M $CaCl_2$ was added. Phage of known titre sufficient to give an average adsorption of 2 particles per cell was then added and aeration continued. Adsorption of T5 was slow even under optimal conditions, but it was possible to obtain multiple infection of 5×10^8 cells/ml. by adding to 100 ml. of cells 10 ml. of phage of a titre of $1{\cdot}7 \times 10^{10}$ /ml. At various times after addition of the phage 10 ml. aliquots were withdrawn and spun for 4 minutes at 3000 r.p.m. in an angle-head centrifuge. A sample of the supernatant was taken for a count of unadsorbed phage and the remainder of the supernatant drained off. It was impossible, however, to remove all the supernatant, and time did not permit washing the cells to eliminate all unabsorbed phage. It was estimated that from 0·1–0·2 ml. of supernatant remained in the centrifuge tubes. For this reason unadsorbed phage could be detected in the samples taken early in the latent period. The cells from the 10 ml. sample were re-suspended in 1 ml. of broth and 0·1 ml. of 0·2 M sodium cyanide added immediately. The tubes were put in ice water for 30 minutes and then into the water bath at 37° for a further 45 minutes. After lysis had taken place they were lightly spun to remove any precipitate of bacterial débris or insoluble salts and the infective phage content of the supernatants determined.

Finally complement-fixation tests were carried out with the cyanide lysates, using falling antigen dilutions and a constant serum dilution of 1 in 8.

Preliminary experiments had shown that, in the medium used, T5 had a minimum latent period of 42 minutes, that the one step growth curve was completed in 65–70 minutes, and that the average burst size varied from 100–200 virus particles per cell. In the cyanide-lysis experiments, therefore, a final sample was taken at 80 minutes for estimation of the virus yield per cell. In satisfactory experiments visible clearing of the culture was always observed before this time.

The manipulations involved in removing the samples, centrifugation and re-suspension in cyanide were carried out at room temperature (usually 25°–27°), and took from 5–7 minutes. There was probably some lag in phage growth at this temperature compared with that at 37°. In presenting the results, the time at which the infected cells were placed in cyanide has been taken as the actual time in the latent period at which phage multiplication was interrupted, but it should be realized that a lag in growth amounting to 3–4 minutes had possibly occurred.

One further comment may be made on the adsorption of T5 in these experiments. It was found that although there was still a large concentration of unadsorbed phage in the cultures 10 minutes after adding the phage, no further adsorption of this free phage occurred. This inhibition of adsorption was observed repeatedly, and although no satisfactory explanation of the phenomenon can yet be offered, it had obvious advantages in that cessation of phage adsorption did permit equilibration of the phage-cell system during the one step growth cycle and prevented re-adsorption on to bacterial débris of infective phage released by cyanide lysis.

TABLE IV.—*Infective Phage and Phage Antigen recovered by Cyanide Lysis at Various Times in the Latent Period.*

Minutes after phage addition.		Phage adsorbed/ 10 ml. cells.	Cyanide lysates.	
Time of taking sample.	Time into cyanide.		Infective phage/ 10 ml. cells.	Antigen titre/ 10 ml. cells.
5	12	$7{\cdot}5 \times 10^9$	$1{\cdot}2 \times 10^8$	6
10	17	$9{\cdot}5 \times 10^9$	$5{\cdot}5 \times 10^8$	4
$17\frac{1}{2}$	24	$9{\cdot}4 \times 10^9$	5×10^8	90
20	28	$8{\cdot}9 \times 10^9$	4×10^9	256
35	40	$9{\cdot}3 \times 10^9$	6×10^{10}	384

Initial cell count = $4{\cdot}5 \times 10^8$/ml.
Final phage count at 80 minutes = $4{\cdot}7 \times 10^{10}$/ml.
Average yield of phage/cell = 96.

Results.

The results obtained in a typical experiment are given in Table IV. In this experiment cells were removed at 5, 10, $17\frac{1}{2}$, 20 and 35 minutes. The amount of phage adsorbed was calculated as the difference between that originally added and that found unadsorbed in the samples. In the samples removed after 10 minutes no further increase in the amount of phage adsorbed was detected. The mean input of phage was therefore $9{\cdot}3 \times 10^8$ particles/ml. on to $4{\cdot}5 \times 10^8$ cells/ml., giving an average infection of 2 particles per cell. The actual amount of phage adsorbed by each 10 ml. sample of cells was therefore $9{\cdot}3 \times 10^9$, which from previous calculations should give complement fixation at an antigen dilution

of 1 in 16. The results show, however, that the samples removed at 5 and 10 minutes and placed in cyanide at 12 and 17 minutes gave antigen titres of only 1 in 6 and 1 in 4. Reasons have already been given for the probability that these titres were due to bacterial antigen rather than to phage antigen. Whether this was so or not, it was obvious that phage antigen could not be recovered from the cells in this part of the latent period in amounts to be expected if the adsorbed phage had retained its antigenicity.

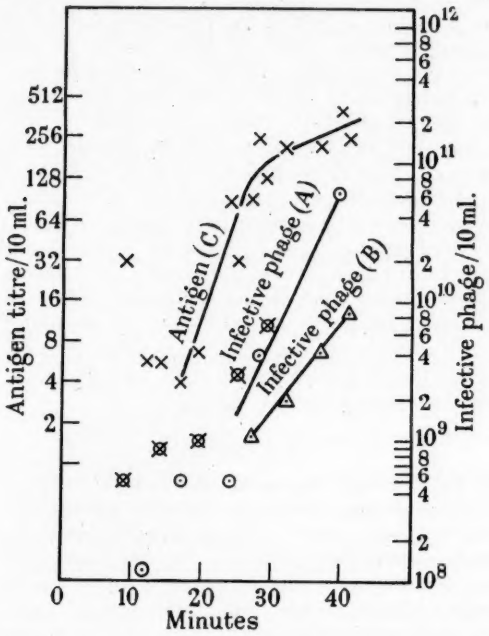

FIG. 4.—Titres of phage antigen and infective phage recovered from T5 infected *Bact. coli* cells lysed with cyanide at various times during the latent period. Data of 3 experiments. Curve A : infective phage recovered from cells with high average burst size (2 experiments). Curve B : infective phage recovered from the cells with low average burst size (1 experiment). Curve C : Combined curve of antigen titres obtained in 3 experiments.

From the 5, 10 and 17½ minute samples small amounts of infective phage were recovered, but the presence of unabsorbed phage in the residual supernatants after centrifuging the samples accounted for these amounts.

In the sample transferred to cyanide at 24 minutes the antigen titre had risen to 1 in 90, although no infective phage could be recovered from these cells. Infective phage at a titre of 4×10^9/10 ml. was detected at 28 minutes and by this time the antigen titre had risen to 1 in 256. The sample taken just before the end of the latent period yielded 6×10^{10} infective phage particles/10 ml. and an antigen titre of 1 in 384. The final phage count at 80 minutes was $4{\cdot}7 \times 10^{10}$/ml., giving an average burst size of 96 particles/cell.

The results of 3 separate experiments are combined in graphic form in Fig. 4.

The antigen titres obtained in the 3 experiments have been plotted on one curve, the scale of which is arranged so that the antigen titre is directly comparable with that of infective phage. Two curves are given for infective phage. Curve A represents the amounts of infective phage recovered in 2 experiments in which large burst sizes (96 and 250) were obtained. Curve B represents the amounts of infective phage recovered in an experiment in which the average burst size was 25. In this experiment the viable cell count at the time of infection was $7{\cdot}8 \times 10^8$/ml., and it appeared therefore that a poor yield of infective virus was obtained from a culture in which the cells were approaching the end of the log phase. The antigen titres, however, were similar to those obtained when yields of infective phage were high. Within 9 minutes or less of addition of the phage no infective phage could be recovered from the cells, but the adsorbed phage had not yet lost its ability to react as an antigen. From 12–19 minutes neither infective phage nor phage antigen could be detected in significant amounts in the cyanide lysates. From 19 minutes onwards the amount of antigen increased at a rapid rate until 29 minutes after the initiation of infection. After this point there was no great increase in the antigen titre, the curve flattening out in the last 10 minutes of the latent period. The appearance of infective phage in detectable amounts was delayed until at least 6 minutes after the time at which the rise in phage antigen began. At no time during the latent period was the quantity of infective phage obtained from the cells sufficient to account for the amount of antigen detected. However, the amount of antigen present in the cells just before the end of the latent period agreed well with the final yield of infective virus at the end of the one step growth curve.

DISCUSSION.

Complement-fixing antibody to phage T5 has been demonstrated in a rabbit serum. The actual conditions necessary for the production of the antibody have not been defined, but it seems of some significance that it was found in a serum of low phage-neutralizing activity. By contrast, other sera containing a high neutralizing titre for other phages of the T series showed no complement-fixing action. Similar behaviour was found when sera of high neutralizing titre for staphylococcal phages were examined. With none of these could complement fixation with their homologous phages be demonstrated. In the past the attentions of those studying phage-antiserum reactions have been directed to obtaining high titre neutralizing antisera, and it seems that such sera may be unsuitable for use in complement-fixation reactions. In such sera union of antigen and antibody may take place too rapidly to allow fixation of complement.

The antigen titre of broth preparations of T5 could be correlated with their titre of infective particles, but it is apparent that antigenicity and infectivity can be dissociated. Phage filtrates in which infectivity had been destroyed by heat or alcohol were still as effective antigenically as was infective phage.

The results obtained by examining T5-infected cells lysed by cyanide led to the postulation of the following sequence of events for this partiuclar phage-cell system. After adsorption on to the cell the infecting phage lost its infectivity before its antigenicity. Antigenicity then disappeared, and for some time neither infective phage nor phage antigen could be recovered from the cells. After 19 minutes had passed the antigen appeared once more inside the cells but infective particles could not yet be demonstrated. The final step or steps which result in

the production of fully infective virus were not completed until after the antigen had been formed. After 30 minutes the cells had apparently almost completed antigen production, and had accumulated a pool of antigenic material from which the infective particles were being organized. In cells which were reaching the end of the logarithmic phase at the time of infection and from which low average burst sizes were obtained, the amount of antigen formed was sufficient to provide a much higher yield of infective virus than was actually obtained. This suggests that these cells were unable to complete the final step of virus synthesis.

In the light of these results a re-examination of those of Latarjet (1948) is of considerable interest. He studied the increase in resistance to x-rays of *Bact. coli* infected by T2r+, and his data indicate the formation, starting at 7–9 minutes, of units which are capable of going on to become infective phage. After 13 minutes the formation of these units stops. As pointed out by Cohen (1949), Latarjet's units cannot be complete T2 virus particles since the latter do not appear until some minutes later in the growth cycle, but their formation does coincide approximately with the beginning of the deposition of desoxyribonucleic acid (DNA), and ends when the amount of protein formed is equivalent to that necessary to complete the virus finally liberated from the cell. Unfortunately, no detailed chemical studies of the course of DNA and protein synthesis in T5-infected cells are available such as have been made for the T2, T4 and T6 virus systems. However, it is not unreasonable to assume that the course of synthesis is similar to T5, having due regard for the longer latent period of this virus. The conclusion is irresistible that Latarjet's units are comparable to the material which, in the T5 system, reacts antigenically, which appears a little less than half-way through the latent period and which precedes the formation of fully infective virus. It may be further postulated that this antigenically-active material corresponds to the phage protein-DNA complex.

The results obtained in the early part of the latent period also provide further evidence in support of the hypothesis of the disintegration or at least partial disruption of the invading virus particle. They indicate a stepwise loss, first of infectivity followed by loss of antigenicity. The loss of antigenicity may in fact be due to an alteration in the prosthetic groups of the antigen which could well accompany a rearrangement of the virus protein or the dissociation from the virus protein of its DNA.

This study of phage growth by means of the complement-fixation reaction has suggested a course of events similar to those indicated by the application of the same method to the growth of influenza virus, and offers further evidence in favour of the essential similarity of virus production in both animal and bacterial cells.

SUMMARY.

The serum of a rabbit immunized with *Bact. coli* phage T5 contained a specific antibody capable of complement-fixation with culture filtrates containing T5. Maximal antigen and antibody titres were determined. No zone phenomena or optimal antigen and antibody titres were found. The antigen titre could be correlated with the titre of infective phage present in the phage preparations. However, infectivity and antigenicity could be dissociated, since phage inactivated by heat or alcohol retained its antigenicity. There were minor cross-reactions

between the serum and other phages of the T series, but these were probably due to a small amount of antibacterial antibody in the serum.

The course of phage multiplication in infected cells was followed, complement-fixing antigen and infective phage being released from the cells by cyanide lysis during the latent period. The invading phage particle lost first its infectivity followed by its antigenicity. Then followed a period, from 12–19 minutes after infection, during which neither infective virus nor antigen could be recovered from the cells. From 19–29 minutes a rapid increase in phage antigen occurred, but completed infective phage did not appear until about 6 minutes after the commencement of this rise in antigen titre. The last quarter of the latent period (30–40 minutes) appeared to be chiefly occupied by the completion of infective virus from the pool of antigenic material.

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THE EFFECT ON EXPERIMENTAL TUBERCULOSIS OF THE INTRAVENOUS INJECTION OF MICROCOCCIN.

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EXPERIMENTS in which carbon was injected intravenously into tuberculous rabbits showed that in certain circumstances carbon was dispersed throughout the caseous centres and epithelioid cells of growing tubercles, and that around the lesions of the spleen and liver there were numerous phagocytic cells containing carbon, though epithelioid cells within the tubercle did not appear to take up carbon directly from the blood stream (Markham and Florey, 1951).

Micrococcin (Su, 1948), an antibiotic only slightly soluble in water, can under certain conditions be dispersed as a finely particulate suspension suitable for intravenous injection. The slight reaction following the introduction of this material into the body has been previously described (Markham, Heatley, Sanders, and Florey, 1951).

In the experiments to be described observations were made on the sensitivity of tubercle bacilli to micrococcin, and on the progress of tuberculosis in rabbits and guinea-pigs, infected respectively with bovine and human tubercle bacilli and given intravenous injections of micrococcin.

EXPERIMENTAL.

The action of Micrococcin on the Tubercle Bacillus in vitro.

It was reported by Su (1948) that the growth of *Myco. tuberculosis*, human type, was completely inhibited by approximately 6·25 μg. of micrococcin per ml. Attempts to repeat and extend this observation were hindered by the relative insolubility of micrococcin. Thus when 50 μg./ml. or more is present in any fluid medium, a haze which is sufficient to obscure the growth of the tubercle bacillus develops at once or after a short time. Difficulties of interpretation also arise, for in considering the minimum inhibitory concentration of an antibiotic the " concentration " generally refers to the amount in solution. With relatively insoluble substances such as micrococcin, other factors have to be taken into consideration. For example, is the effective concentration determined by the amount strictly in solution, or can some or all of the undissolved substance help to exert an antibacterial effect ? It is easy to imagine—and there is some experimental evidence in favour of the view—that the bacteria take up micrococcin as one step in the process of being inhibited. The ambient solution would thus become unsaturated and could dissolve more micrococcin, and it is possible that many times the amount of micrococcin that was in solution initially could be taken up by the bacteria in this way. The effective concentration of micrococcin

would thus be greater than that strictly in solution, though probably less than the total amount present. It might be expected to depend on many factors additional to those normally determining the activity of a soluble antibiotic. Among these additional factors might be degree and uniformity of dispersion of the solid ; extent to which settling under gravity could occur ; extent of Brownian movement or gross agitation, if any ; temperature ; rate of solution ; and presence or absence of a layer of less soluble material on the surface of each particle of micrococcin. This last may be an important factor, for it is known that the surface of solid micrococcin becomes converted to a yellow (? insoluble) product under the action of oxygen and light (Heatley and Doery, 1951). Some of these factors are obviously interrelated, and would be very laborious to investigate.

For this reason the study of the effects of micrococcin on the tubercle bacillus *in vitro* was only pursued far enough to indicate whether the experiments *in vivo* that are reported in this paper would be worth doing.

Effect of micrococcin on dispersed growth in fluid medium.

Several strains of *Myco. tuberculosis* were tested in the medium of Dubos and Davis (1946) containing two-fold falling dilutions of crystalline micrococcin made up originally in alcohol. The small concentration of alcohol finally present was shown to have no effect on the growth of the tubercle bacillus. The results, summarized in Table I, show that the growth of several strains of the organism is inhibited by micrococcin.

Effect of micrococcin particles on surface growth on solid medium.

Several series of experiments were done, using either Kollé flasks or petri dishes containing Dorset's egg medium or Löwenstein-Jensen medium and a variety of dyes. Both human and bovine strains of tubercle bacilli were used. The procedure was to flood the surface of the medium with a suspension of the tubercle bacillus. After overnight incubation the excess moisture was removed,

TABLE I.—*Results of Dilution Tests with Crystalline Micrococcin against Various Strains of* Myco. tuberculosis. *Dubos and Davis Fluid Medium was used throughout.*

Strain.	Days incubated.	Concentration of micrococcin (μg./ml.) giving—		
		No inhibition.	Partial inhibition.	Complete inhibition.
H37Rv	4	0·8	1·6, 3·1	6·2
	10	0·8	1·6, 6·2	12·5
H37Rv	4	0.8	1·6–12·5	–
	10	3.1	6.2, 12·5	–
Human PN	4	0·8	1·6, 3·1	6·2
	10	3·1	6·2	12·5
Human DT	4	0·2	0·4, 0·8	1·6
	10	3·1	6·2	12·5
Bovine Branch	4	0·2	0·4–1·6	3·1
	10	0·8	1·6, 3·1	6·2
Bovine Branch	4	0·8	1·6–12·5	–
	10	1·6	3·1–12·5	–
Bovine S/182	4	1·6	3·1	6·2
	10	0·4	0·8–3·1	6·2

loopfuls or drops of micrococcin suspension were placed on the surface of the medium, and the vessel was sealed and returned to the incubator.

There was inhibition of growth in, and immediately around, the area of the drop, the rest of the surface of the medium being covered with more or less confluent growth (Fig. 3). A film made from the areas of inhibition showed some acid-fast bacilli, many of which were elongated and showed fragmentation. Out of sixteen cultures made from areas where no growth was apparent, eleven were sterile, one bore a single colony, one a few colonies, and three many colonies. Too much stress should not be placed on these results, for on the one hand it was not always easy to avoid the area outside the drop where there was a good growth, and on the other hand particulate micrococcin might have been taken up with any viable bacteria and transferred to the new medium. There is no doubt, however, that the growth of all the strains of tubercle bacillus that were tested in this way was inhibited.

Further tests were done using the oleic-acid albumen agar medium of Dubos and Middlebrook (1947). Loopfuls of micrococcin suspension were placed on the surface of the medium after overnight incubation, as described above. All the plates showed a zone of inhibition around the micrococcin drops. With the human type of tubercle bacillus the zone ranged from 7 to 12 mm. in width. Within the zone of inhibition there were, however, a few colonies of bacilli. Cultures from these colonies were made in the fluid medium of Dubos and Davis (1946), and were used in further plate tests as above. After incubation there was no zone of inhibition round the drops of micrococcin suspension, suggesting that resistance to micrococcin had increased.

Inhibition of surface growth by micrococcin dispersed in the medium.

In these experiments slopes of Dorset's egg medium were intimately mixed with various amounts of a fine suspension of micrococcin inmediately before inspissating, and the surface of the prepared medium was inoculated with a suspension of the organism. The human strain, H37Rv, was markedly inhibited on media containing 25 μg. or more of micrococcin per ml., and a bovine strain was partly inhibited in the early stages of growth by 50 or 100 μg./ml. (It is possible that the micrococcin sedimented slightly during inspissation, so that its concentration at the surface of the medium, i.e., at the site of inoculation of the organisms, may have been rather less than the figures indicate.)

Development of resistance.

It is impossible to ascertain clearly how easily and to what extent resistance to micrococcin is developed by the tubercle bacillus. In liquid medium the inhibitory concentration is only slightly below that at which growth of the organism is obscured by the presence of micrococcin in suspension, so that the increase in resistance which can actually be demonstrated is only 2- or 4-fold—a hardly significant amount. On solid medium presence or absence of growth can be observed whatever the concentration of micrococcin; however, the fact that growth does occur on a high total concentration of micrococcin does not necessarily mean that the organism has become highly resistant, since the effective concentration of micrococcin may not be much greater than that actually in solution. It seems probable that an organism which will grow on a certain total

concentration—probably less than 50 μg./ml.—will grow on any concentration of micrococcin. Although the development of resistance cannot formally be demonstrated, there are indications and analogies which suggest that the tubercle bacillus does become readily resistant. Thus, as mentioned above, zones of inhibition around particles of micrococcin could be demonstrated on the surface of solid medium. When the occasional colonies found inside these zones were used for seeding fresh test plates, no zones of inhibition were then formed.

Among more sensitive organisms such as the staphylococcus a high degree of resistance develops rapidly in liquid midium. Experiments on solid medium with the same organisms showed two kinds of resistant colony : (1) an occasional colony developing without any increase in the lag period on any concentration of micrococcin ; these colonies were highly resistant ; and (2) much more numerous colonies which appeared only on a just inhibitory concentration and grew only after a prolonged lag period ; these were only slightly resistant to micrococcin. Colonies of resistant tubercle bacilli have been observed to grow up both with and without an increased lag period. If the conclusion of Bryson and Demerec (1950) is valid, that for any given antibiotic the pattern of the development of resistance is the same for most organisms, then it may be inferred that the tubercle bacillus also will acquire a high degree of resistance.

The ease with which resistance to micrococcin is developed by various bacteria would at first sight seem a fatal obstacle to its therapeutic use, but though resistance to streptomycin develops rapidly, that drug has undoubted clinical value.

THE TREATMENT OF TUBERCULOUS RABBITS WITH MICROCOCCIN.

All rabbits were inoculated intravenously in an ear vein with 0·01 mg. of tubercle bacilli, strain " Branch." This is a fully virulent strain of bovine type. Five days later intravenous injections of suspensions of micrococcin prepared as previously described (Markham *et al.*, 1951) were begun in half the animals. This period of 5 days between the time of inoculation and the beginning of treatment was chosen as a result of observations made on the dispersion of carbon injected intravenously in the lesions of tuberculous rabbits (Markham and Florey, 1951). Injections were given daily for from 5 to 20 days.

Two rabbits received 5 equal daily injections totalling 100 mg. and 500 mg. respectively. The first animal died 27 days and the second was killed in fair condition on the 37th day after infection. Three other animals were treated as follows : One received a total of 1000 mg. of micrococcin in equal daily doses over a period of 10 days. This rabbit died 28 days after infection, and 14 days after the last injection of micrococcin. Another received a total of 1300 mg. of micrococcin in 14 equal daily doses, and died 19 days after infection (1 day after the last injection). The third was given 20 equal daily injections totalling 2000 mg., and became sick and was killed 25 days after infection (the day after the last injection.)

A control untreated animal was killed with each treated animal, and pieces of spleen, liver, lung and kidney were fixed in formol-saline for histological purposes, while other pieces were autoclaved, and assayed for micrococcin content.

Fluorimetric Estimation of Micrococcin in Tissues.

Pieces of tissue taken at post-mortem examination from tuberculous animals were autoclaved, then weighed portions were extracted with alcohol and the

fluorescence of the extract measured (Heatley, Kelly and Smith, 1951). The micrococcin content of certain organs of the group of three tuberculous rabbits

TABLE II.—*Content of Micrococcin (in Terms of Fluorescence of Alcoholic Extracts) of Autoclaved Organs of Tuberculous Rabbits.*

No.	Total dose of micrococcin (mg.)*.	Interval (days) between death and— first dose.	Interval (days) between death and— Last dose.	Concentration of micrococcin, μg./g., in— Liver.	Spleen.	Lung.	Kidney.
1	1300	14	1	2250	567	1830	573
2	2000	20	0	3960	5060	2320	–
3	1000	23	13	900	840	310	390

*In consecutive daily doses of 100 mg.

mentioned above is given in Table II. The actual concentration was probably higher than that observed, for it was shown in a non-infected animal that autoclaving reduced the apparent micrococcin content. There is without doubt a substantial concentration of the antibiotic in the organs containing tuberculous lesions, the relative abundance in different tissues agreeing with the assessments made by histological examination.

Observations on the Living Animals and Macroscopic Post-mortem Appearances.

None of the rabbits appeared to suffer in any immediate way from the injection of the suspended material. Neither treated nor untreated animals showed any signs of disease until about the 16th day after infection. From that time onward a fairly steady loss of weight occurred in them all, sometimes becoming very marked during the few days before death. In all except one pair the weight of the treated animal fell more rapidly than that of its control, and in every case the treated animal suffered greater terminal respiratory embarrassment and died before the control animal.

Post-mortem examination showed that the lesions in the lungs, liver and kidneys of the control animals were larger and more numerous than in those of the treated animals, although the lungs of the latter were rather more solid in consistency. The lesions in the rabbits which had received large doses of micrococcin did not differ to any significant extent from those in animals which had had smaller doses. The only exception was in the rabbit which had received the smallest dose (100 mg.), in which no modification was observed in the tuberculous process compared with that in controls. Only one of the treated animals had macroscopic lesions in the liver, while " pin-point " liver lesions were present in all the controls. The macroscopic appearances of the spleens were so varied that no conclusion could be drawn from them.

Histological Examination.

Paraffin sections were prepared from formalin-fixed material, and the distribution of micrococcin was observed in frozen sections of the same material by ultra-violet light (Markham *et al.*, 1951).

Lungs.

In the *control animals* the essential lesion was a mass of epithelioid cells which might or might not contain a central area of caseation. The zone of epithelioid

cells was always wide, and lymphocytes were usually fairly numerous among the peripheral cells. The tubercles tended to become confluent, when they formed large masses of tuberculous tissue containing areas of caseation.

In the animals *treated with micrococcin* the three most striking features were the small number of discrete or typical tubercles, the small amount of caseation and the small area of lung tissue involved (cf. Fig. 1 and 2.) The commonest lesion consisted of a diffuse cellular tissue which was present in compact masses and formed cords and groups of cells filling the alveolar spaces (Fig. 4). Small areas of necrosis and of caseation might be present in these cell masses. The epithelioid cells were large and well defined, while lymphocytes were few.

The greater the time between inoculation and death the more alike did the lesions of control and treated animals become, and this was noticed even in animals which had received micrococcin injections up to the time of death.

The reason for the greater respiratory embarrassment noted in the treated animals is obscure. It is conceivable that the blood flow in the tuberculous lung was impeded by the presence of thrombi containing micrococcin in the finer vessels, but there was no clear evidence that this was so.

Spleen.

In the *control animals* many discrete circular tubercles were scattered throughout the sections and were separated by normal splenic tissue. The older lesions were becoming confluent. The architecture of each of the tubercles was fairly typical, and many of them appeared to have developed in and to have replaced the splenic lymphoid nodules, traces of which could still be seen. Caseation was a conspicuous feature.

In the *treated animals* the splenic tissue was almost completely replaced by a diffuse tuberculous tissue in which it was difficult to recognize a typical tubercle. The lesion was made up of cords and sheets of epithelioid-like cells, which seemed to be spreading throughout the organ. The epithelioid cells were large, well defined, and rather more regular in size and shape than those of the untreated animals. They had an abundant cytoplasm full of minute vacuoles, giving the appearance of a central pale area surrounded by a more densely staining rim of cytoplasm. The nucleus, which was fairly regular and open, was at one side of the cell. Areas of necrosis and caseation, though present, were few and small.

It was possible to distinguish tuberculous epithelioid cells from macrophages which contained micrococcin granules. The macrophages tended to lie in groups and to stain less densely than the epithelioid cells; the closely packed vacuoles were larger and more irregular in size; the nucleus was denser and often flattened and crescentic. Epithelioid cells could be seen adjacent to micrococcin-containing macrophages.

Kidneys.

The lesions in the *control animals* consisted of numerous scattered, well circumscribed tubercles situated in the cortex; it was rare to find a lesion in the medulla. In all rabbits the lesions varied in size, and it was possible to find very early lesions occupying part of a glomerulus. The larger lesions had a central area of caseation and a peripheral zone of lymphocytes.

In the *treated animals* the small number and size of the kidney lesions as compared with controls was their most noticeable feature. The differences in histo-

logical appearances were not as marked as in the lung and the spleen. The lesions situated in the cortex, which showed considerable variation in size, did not have the circumscribed contour seen in lesions in the control animals, and contained little caseation.

As the lesions increased in size more glomeruli became involved, and from observations made with carbon (Markham and Florey, 1951) it is probable that the particulate micrococcin appearing in the tubercles came from material arrested in the glomerular vessels.

Liver.

The lesions of the *control animals* were small, discrete and scattered throughout the liver. Many of them developed in or near the portal tracts, but others were found at various situations in the hepatic lobules. Central caseation was a common feature, and in most of the lesions a sparse lymphocytic infiltration and some condensation of reticulin or even fibroblastic activity was present at the periphery. These features combined to give the characteristic appearance of a tubercle with a sharply defined outline (Fig. 5).

The lesions of the *treated rabbits* showed some differences from those of the controls. Generally the amount of tuberculous tissue was greater in treated than in control animals. The lesions showed little caseation, but small areas of recent necrosis were sometimes present. The sharp outline of the tubercles seen in the control animals was absent; instead, the tuberculous tissue had spread into the surrounding liver tissue in the form of irregular clumps of cells (Fig. 6). Lymphocytes were almost entirely absent, and there was no peripheral reticulin condensaton or fibroblastic activity. The epithelioid cells were similar to those observed in lesions elsewhere.

Distribution of Micrococcin in the Tissues of Tuberculous Rabbits.

The distribution of micrococcin was followed by the examination of frozen sections in ultra-violet light (Markham *et al.*, 1951).

Lung.—Fluorescent particles were plentiful in the alveolar walls, and in broncho-pneumonic areas the alveolar pattern could be vaguely traced by the distribution of the particles. The course of completely obliterated and partly degenerated vessels was shown by cords of micrococcin-containing cells. Very few particles were present in areas of compact tuberculous tissue, and they were entirely absent from areas of caseation and necrosis except when a vessel happened to pass through a caseous area.

Spleen.—Sections of spleen showed anastomosing cords and groups of cells filled with brightly fluorescent particles of micrococcin. This fluorescent latticework passed between non-fluorescent areas of tuberculous tissue or the remains of Malpighian bodies (Fig. 8). Some fluorescent material was occasionally seen in cells situated among the peripheral epithelioid cells of the lesions. The large amount of micrococcin present in the splenic tissue and its almost total absence from the tuberculous lesions in this organ was very striking.

Liver.—The dispersion of micrococcin throughout the liver was very even, except for irregular areas of tuberculous tissue which were devoid of fluorescent material (Fig. 7). Only in the outer edge of such areas was it possible to identify

any micrococcin, and this seemed to be contained in Kupffer cells which had become involved in the tuberculous lesions.

Kidneys.—The tuberculous lesions were free from micrococcin except where a glomerulus containing the substance had become involved. In this case particles could still be seen in the remains of the glomerulus.

Investigation for Tubercle Bacilli.

Acid-fast bacilli were present in the lesions of both treated and untreated animals. In both the bacilli were most numerous in or about caseous areas, and they were relatively uncommon in the diffuse tuberculous tissue which was the chief lesion in the treated animals. Clumps of bacilli were less common in treated than in untreated animals, and there was a general impression that the total number of tubercle bacilli was less, though the difference was not striking.

Residual Activity of Micrococcin.

That the micrococcin present in the lung and liver was still active was shown by placing on agar plates seeded with staphylococci portions of crushed tissue from a tuberculous rabbit that had received 1000 mg. of micrococcin. The animal had died 28 days after the last injection. After incubation a zone of inhibition was found round the tissue.

EXPLANATION OF PLATES.

FIG. 1.—Lung of a control rabbit killed 24 days after inoculation. Extensive areas of confluent tuberculosis, in which caseation is marked, are present. × 12.

FIG. 2.—Lung of a rabbit which had received 2000 mg. of micrococcin, and which was killed 24 days after inoculation while it was still receiving micrococcin. Some fairly typical tubercles are present and there are small areas of caseation. × 12.

FIG. 3.—Kollé flask containing a culture of *Myco. tuberculosis*, human type H37Rv, on Löwenstein-Jensen medium. Six drops of micrococcin suspension were placed on the medium and the flask incubated for 52 days at 37°. There is complete inhibition of growth in the areas over which the drops have spread.

FIG. 4.—The cellular type of tuberculous lung lesion found in the treated rabbits. Note the completely obliterated vein in the central part of the field. Haematoxylin and eosin. × 100.

FIG. 5.—A circumscribed tuberculous lesion in the liver of a control rabbit which was killed 28 days after infection. Note the caseous central area and the peripheral zone of lymphocytes. Haematoxylin and eosin. × 100.

FIG. 6.—A tuberculous lesion in the liver of a rabbit which had received 1000 mg. of micrococcin. Note the cellular proliferative type of lesion and the absence of caseation. The rabbit was killed 28 days after infection. Haematoxylin and eosin. × 100.

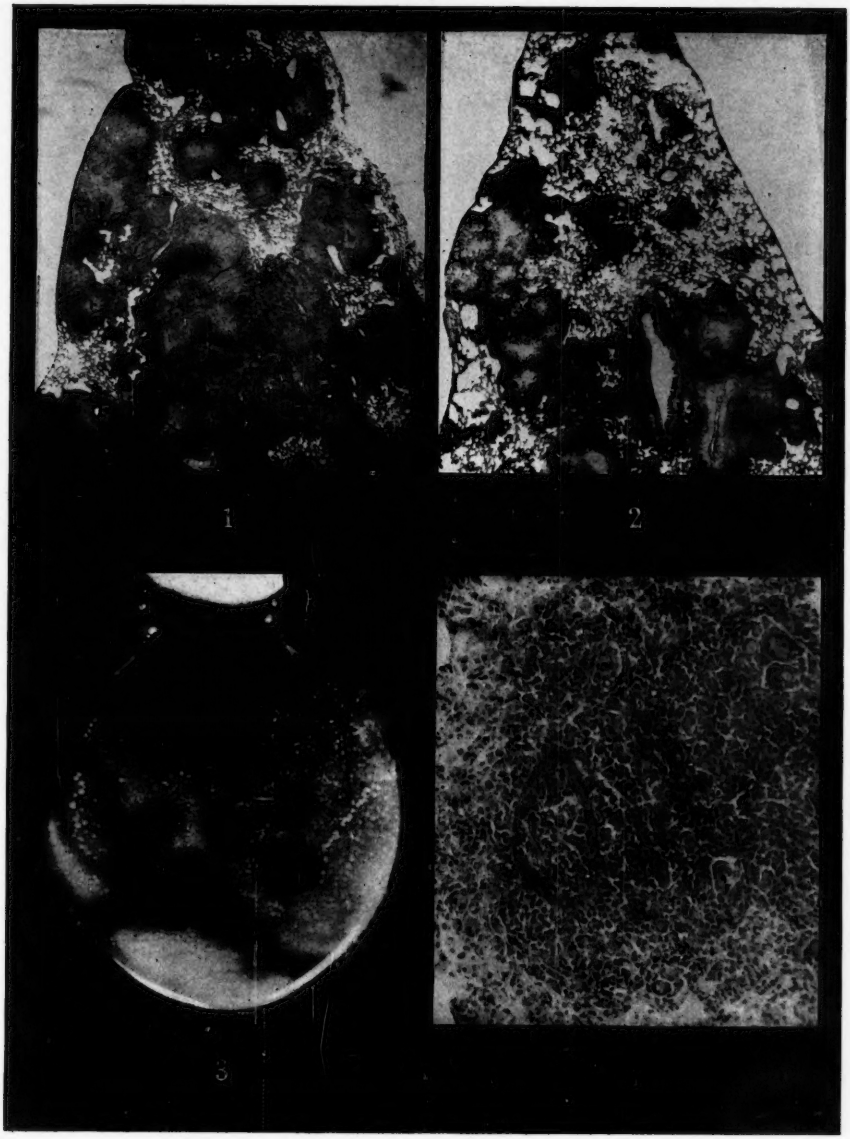
FIG. 7.—A tuberculous lesion in the liver of a rabbit which had received 1300 mg. of micrococcin and which died 19 days after infection. Large amounts of micrococcin can be seen in the liver tissue but none can be seen in the tuberculous lesion. Haematoxylin: ultra-violet light. × 100.

FIG. 8.—A tuberculous lesion in the spleen of a rabbit which had received 2000 mg. of micrococcin. Large amounts of micrococcin can be seen in the tissue which surrounds the lesion but none in particulate form can be recognized in the lesion. Haematoxylin: ultra-violet light. × 100.

FIG. 9.—Portions of the spleen of a treated guinea-pig (right) and a control animal (left). The treated guinea-pig had received 280 mg. of micrococcin. Both were killed 25 days after infection. × 1·7.

FIG. 10.—The spleen of a control guinea-pig killed 25 days after an intramuscular injection of tubercle bacilli. Most of the Malpighian bodies have been replaced by tuberculous lesions which are spreading into the pulp. × 12.

FIG. 11.—Spleen of a tuberculous guinea-pig which had received 280 mg. of micrococcin and which was killed 25 days after infection. Some small greyish tuberculous areas can be seen in Malpighian bodies. × 12.



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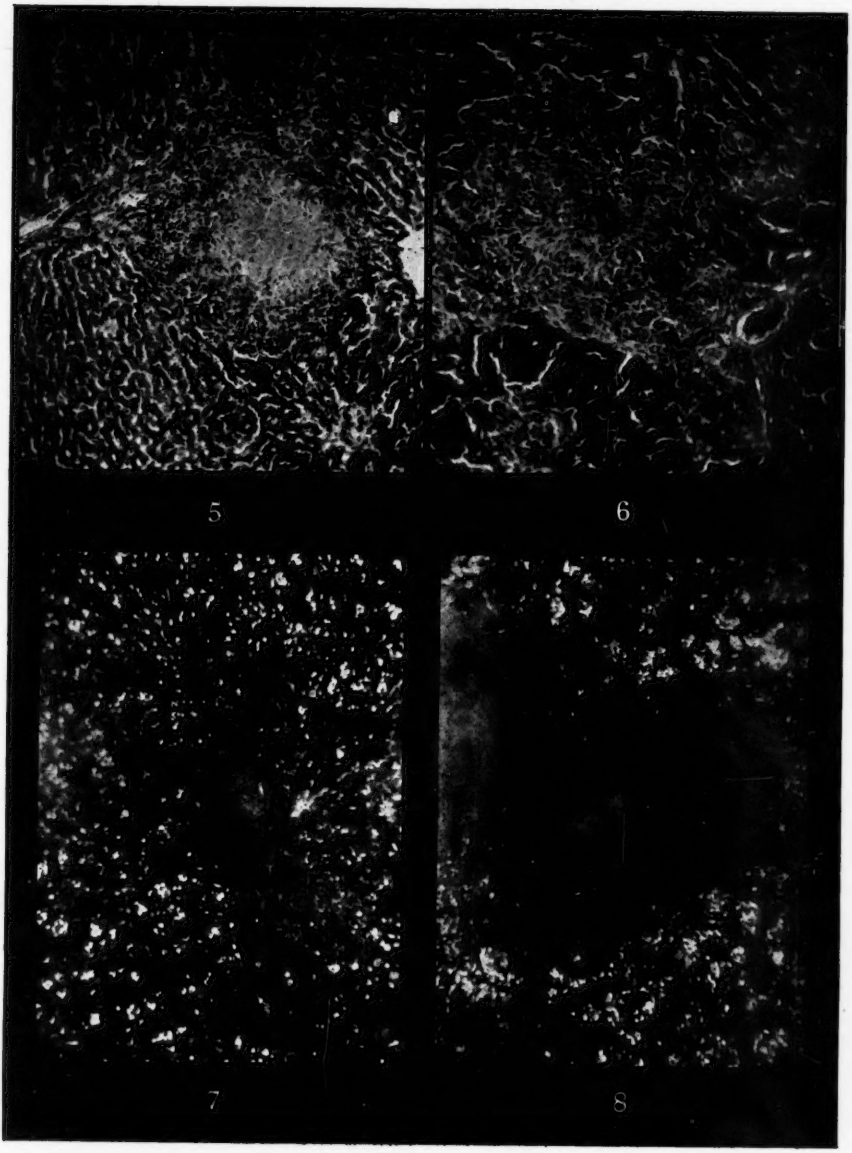

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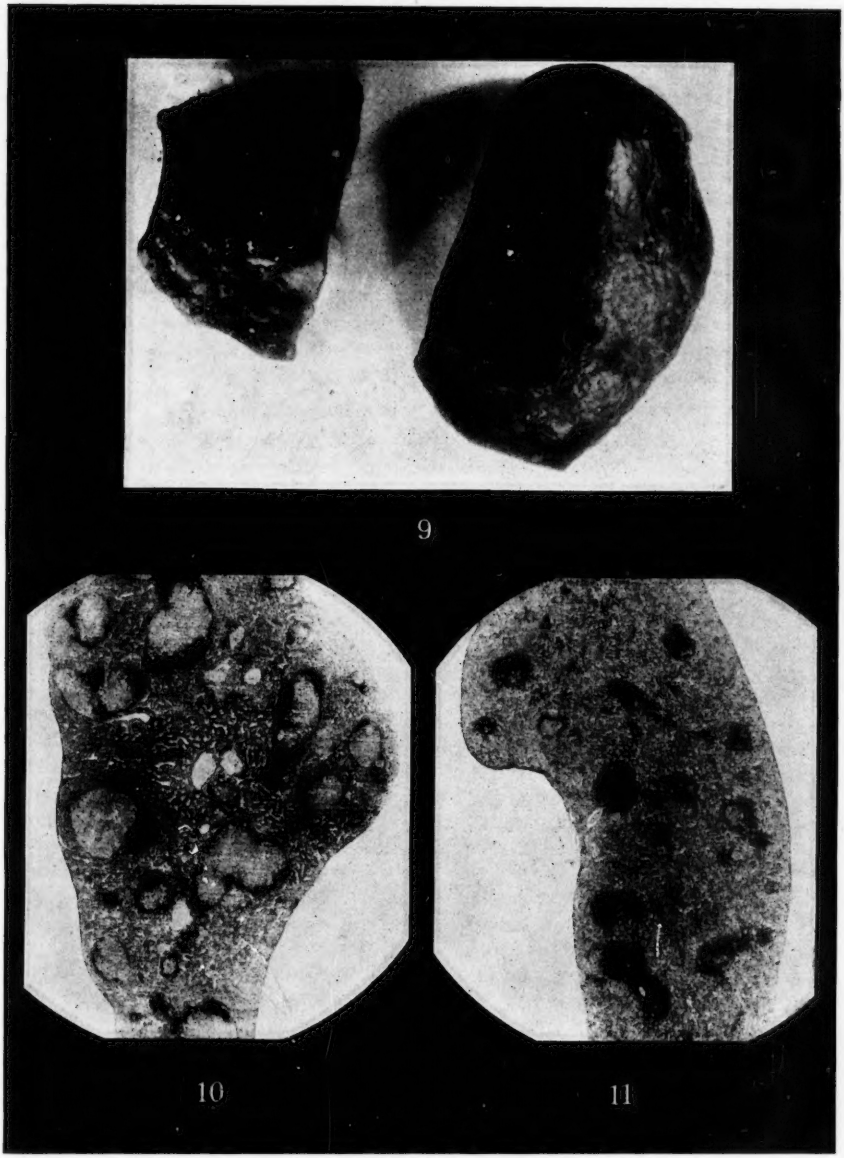

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Markham, Wells, Heatley and Florey.

THE TREATMENT OF TUBERCULOUS GUINEA-PIGS WITH MICROCOCCIN.

Twenty guinea-pigs were inoculated with 0·01 ml. of an 8-day-old culture of human tubercle bacilli (H37Rv), in Dubos and Davis's liquid medium, into the muscle of the right thigh. Ten of the animals were set aside as controls, and the rest formed two groups of 5 for treatment with micrococcin. The first group received 40 mg. and the second 20 mg. of micrococcin intravenously at each injection. The material injected consisted of fine particles of micrococcin suspended in a mixture of saline and guinea-pig serum at a concentration of approximately 20 mg. per ml. The method of injection is described by Markham and Kent (1951). The injections were begun 4 days after inoculation and were repeated every third day. One animal from each treated group and two control animals were killed at 25, 33, 40, 48 and 55 days after infection. The animals from the first group which were killed after 25 and 33 days had received total doses of 280 and 400 mg. respectively, and the rest all received a total dose of 480 mg. Corresponding animals from the second group received half these doses.

Observations on the Living Animals and the Post-mortem Appearances.

Weight.—The weights of the animals at weekly intervals showed no constant variation in either direction and were essentially the same in the control and treated groups.

Mantoux tests.—Eight days after inoculation a Mantoux test with 1 in 10 Old Tuberculin was negative in 6 of the treated and 2 of the control animals. Seven days later and again after a further 10 days, using 1 in 100 Old Tuberculin, all the animals gave a positive reaction, but treated animals generally reacted less strongly than control animals.

Post-mortem examination.—All the animals, both treated and control, were in good physical condition when killed ; in none was there macroscopic evidence of advanced tuberculosis.

All the treated animals had marked splenomegaly ; in the spleens of those killed on the 25th and 33rd days little evidence of tuberculosis could be seen, while those of control animals were studded with tubercles (Fig. 9). Treated animals killed later than this had fairly large tubercles in the spleens, but the lesions were not as extensive as in the corresponding control animals.

In none of the animals killed on the 25th and 33rd days was the liver involved macroscopically, but in those killed later small isolated lesions had appeared, and these were smaller in treated than in control animals.

In one guinea-pig only (a control animal killed on the 25th day) were lesions visible in the lungs. In no case were lesions seen in the kidneys.

The site of inoculation showed a considerable amount of tuberculous inflammatory tissue and some caseation. The regional lymph nodes were enlarged and the normal lymphoid structure was replaced by tuberculous tissue and caseous material. Some of the treated animals had less extensive caseation at these sites than any of the control animals.

Distribution of Micrococcin.

The amount of micrococcin in the autoclaved organs of the treated tuberculous animals was estimated by the fluorimetric method previously described. Table

III shows the results which, because of the known loss of micrococcin from tissue

TABLE III.—*Content of Micrococcin (in Terms of Fluorescence of Alcoholic Extracts) of Autoclaved Organs of Tuberculous Guinea-pigs.*

Experiment No.	No. of doses.	Days between first dose and death.	Total micrococcin (mg.).	Concentration of micrococcin, μg./g., in—			
				Spleen.	Liver.	Lung.	Kidney.
1a	7	21	140	3070	2920	155	38
1b	„	„	280	13650	5230	767	180
2a	10	29	200	3260	3690	404	106
2b	„	„	400	5660	3970	470	130
3a	12	36	240	4700	3300	225	66
3b	„	„	480	6370	13000	550	70
4a	12	44	240	720	1560	105	50
4b	„	„	480	1500	1600	111	75
5a	12	51	240	780	1000	100	27
5b	„	„	480	400	1100	105	62

during autoclaving, will tend to be somewhat low. It is clear that considerable amounts of micrococcin were present in the spleen and liver throughout the experiments.

Histological Observations.

Spleen.

In all the *control animals* tuberculous lesions of various sizes were present in the spleen; in those that were killed on the 25th day the lesions were mostly confined to the Malpighian bodies but a few were spreading out into the red pulp (Fig. 10). Early lesions consisting of a small group of epithelioid cells could often be seen situated in one side of a Malpighian body, and this appeared to be the place where most of the lesions had begun.

In guinea-pigs killed after the 25th day many of the lesions had become confluent and areas of caseation were present; the remains of Malpighian bodies were sometimes seen at the periphery of an area of tuberculous tissue. Some of the caseous areas contained the remains of numbers of polymorph nuclei, and occasionally deposits of calcium were present.

In the *treated animals* the distribution and the general architecture of the lesions were in most respects similar to those in the control animals. It was apparent, however, that the disease process had been slowed, and that this slowing was more marked in those that had received the larger dose of micrococcin.

In the treated animals killed on the 25th day the only evidence of tuberculosis was the presence of small collections of epithelioid cells in some of the Malpighian bodies (Fig. 11). Animals killed later showed more extensive lesions but always smaller and fewer than in the corresponding control animals, and caseation was either small in amount or absent.

Distribution of micrococcin.—Micrococcin was scattered diffusely through the red pulp and occasional granules could be identified in cells in the Malpighian bodies. An occasional cell situated among the epithelioid cells at the periphery of a tuberculous lesion contained particles of micrococcin; apart from this, micrococcin could not be identified in the tuberculous tissue.

Liver.

Tuberculous lesions were fairly numerous in all the *control animals* but involvement was not as extensive as in the spleen. Many of the older lesions were massive and contained small areas of caseation, and collections of lymphocytes were sometimes present at the periphery.

Although tuberculous lesions were present in all the *treated animals* they were smaller and fewer than in the controls, though distribution and cytology were the same. Necrosis and caseation were small in extent and lymphocytes were few.

Distribution of micrococcin.—Micrococcin was absent from the tissue of the portal tract and could not be identified in the tuberculous tissue, but was present in the Kupffer cells of the surrounding hepatic tissue.

Lungs.

In neither the treated nor the control animals were the lungs involved to any extent in the tuberculous process, and no significant difference could be detected between the two series. A few particles of micrococcin were present in the alveolar walls but the amount present was small.

Kidneys.

No lesions were found in the kidneys of either the control or the treated animals.

Very occasional particles of micrococcin were sometimes seen in the loops of the cortical glomeruli.

Lesions at the site of inoculation.

At the site of inoculation in the thigh large irregular masses of tuberculous tissue were situated in the muscle. These consisted of bands of fibrous tissue separating sheets of epithelioid cells in which extensive caseation had sometimes occurred. In some places a wide zone of epithelioid cells surrounded a dense mass of degenerating polymorphs. There was no histological difference between the lesions of treated and control animals. No micrococcin was detected in these areas.

Bacteriological Observations.

When each animal was killed a smear and culture were made from a small portion of the spleen and liver. In every case tubercle bacilli were seen in the smear and grew in the culture.

In paraffin sections tubercle bacilli were found in the lesions of both the control and the treated animals. The bacilli were most plentiful in and about areas of caseation and, since there was more caseation in the control animals, tubercle bacilli were rather more numerous in these than in the treated animals.

DISCUSSION.

The tuberculous lesions in the rabbits and guinea pigs treated by the intravenous injection of particulate micrococcin were at first smaller and fewer than those in control animals, but they were proliferative in type and showed no evidence of regression. In the treated animals there was less necrosis and

caseation, which may have been due to the delayed development of hypersensitivity, since the treated guinea-pigs developed sensitivity to Old Tuberculin somewhat less rapidly than the controls. There was no prolongation of life of the rabbits, in fact some of the treated animals died before the controls. No satisfactory explanation can be offered for this phenomenon. From previous work (Markham *et al.*, 1951) it seems unlikely that it was caused by the toxicity of the micrococcin itself.

The most that can be said is that there was retardation of the early stages of the development of tubercles in organs in which much micrococcin was retained in reticulo-endothelial cells.

A number of reasons can be advanced for the smallness of the effect observed. It may be that micrococcin did not enter the cells of the tubercle in sufficient quantity to inhibit the growth of the bacilli. It will be recalled that carbon administered in the same way as micrococcin was found in the epithelioid cells if it were given within 10 days after infection (Markham and Florey, 1951). It was inferred that micrococcin would behave similarly, but no direct evidence for this was obtained as micrococcin could not be seen in the tuberculous lesions, though it is possible that particles were present but were so finely divided that their fluorescence could not be detected. It has also been noted that very fine particles lose their fluorescent properties rapidly when viewed in ultra-violet light. Even if small amounts did reach the epithelioid cells they may have been " used up," for it has been demonstrated (Su, 1948 ; Gowans, unpublished) that if a subinhibitory amount of micrococcin is added to a culture of a sensitive organism it disappears as the organism grows. Thus tubercle bacilli might actively destroy small amounts present in cells of the tubercle.

It has been postulated that even if a particulate antibiotic could not be made actually to enter a tubercle from the blood stream, a beneficial effect might be exerted if the tuberculous area could be surrounded by a " wall " of macrophages " fortified " by the ingestion of an antituberculous drug (Markham and Florey, 1951). There is no doubt that in both guinea-pig and rabbit the phagocytic cells between the growing lesions in the spleen and to a less extent in the liver contained micrococcin and could be considered to form such a " wall," but, except in the initial stages, the growth of the tubercles was not much impeded. One may infer that the original conception was faulty but, since some transitory inhibitory effect could be shown on tubercles growing in these organs, the possibility remains that other antibiotics introduced into the phagocytic cells might give better results. Micrococcin has certain drawbacks, amongst which is the capacity of organisms to acquire resistance rapidly. The resumed growth of tubercles after early retardation might be due to this, though it has not been possible to demonstrate it. Further, the tubercle bacillus is not very sensitive to micrococcin, much less so than to, for instance, streptomycin.

It seems worth while to pursue the idea of " fortifying " the cells of tubercles with an antituberculous substance injected intravenously, or at least to try to concentrate an active substance in the neighbourhood of the lesions.

SUMMARY.

Micrococcin is soluble only to the extent of about 1 μg./ml., but it can form supersaturated solutions. It inhibits the growth of the tubercle bacillus *in vitro*.

Rabbits infected intravenously with bovine tubercle bacilli were treated by

the intravenous injection of suspensions of micrococcin. The treated animals died somewhat sooner than control infected animals, but their lesions, especially in the early stages, showed greater proliferation of epithelioid cells and less caseation than those of control animals.

In guinea-pigs infected by a human strain of tubercle bacillus the development of lesions in the spleen and liver was retarded but not prevented by micrococcin.

The distribution of micrococcin in tuberculous animals was studied. The drug did not appear to enter the tubercles in significant amount.

We are indebted to the Medical Research Council for grants for technical assistance and materials, and to Mr. B. K. Kelly and his staff, of the Clevedon Antibiotics Research Station of the Medical Research Council, for the fermentation and partial preparation of most of the micrococcin used in this work. We are grateful for the technical assistance given by Mr. J. Kent, Mr. D. W. Jerrome, Mr. N. Smith, Mr. J. Cawston, Mr. C. A. Graham, Miss E. Page, Mr. L. Jeffs and Mr. A. A. Argyle. We are indebted to Dr. M. A. Jennings and Miss W. M. Poynton for help in the preparation of this paper.

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A TECHNIQUE FOR THE REPEATED INTRAVENOUS INJECTION OF GUINEA-PIGS.

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IN the past guinea-pigs have seldom been used in experiments which have required the injection of substances intravenously. In the experiment recorded by Markham, Wells, Heatley and Florey (1951) a method was used which made it possible to give a relatively large number of intravenous injections into the ear vein of a guinea-pig. An intravenous injection can be made into the ear vein with ease if two conditions are satisfied. Firstly, the ear must be immobilized on a flat surface, and secondly, it must be illuminated in such a way that the vessels can be seen clearly. The apparatus used is shown in Fig. 1. It consists of an ordinary class microscope lamp, a retort stand fitted with a clamp carrying an internally reflecting rod, a syringe fitted with a No. 27 gauge, long bevel needle, and a pair of magnifying spectacles. The rod used was made of perspex ; on the top of its short vertical arm a perspex disk 2·5 cm. in diameter was cemented with perspex dissolved in chloroform. It was an easy matter to spread the ear of a guinea-pig over this disk and to fix it flat. The rod transmitted sufficient light to make the vessels clearly visible.

The hair was clipped from about the ear of the guinea-pig, and if necessary the outer surface was shaved. The unanaesthetized animal was wrapped in a towel in such a way that the head and ears protruded, and was held by an assistant in a position that allowed one ear to rest evenly on the lighted disk. The assistant then held the guinea-pig in position with one hand while with a finger of the other he fixed one side of the ear ; the opposite side of the ear was fixed by a finger of the operator. The needle was then inserted gently into the chosen vein and the operator's steadying finger shifted and placed over the needle above its point of entry into the vein (Fig. 2). It was found that the smaller veins were easier to enter than the larger veins, and in ears where a good marginal vein was present this was always the vein of choice. The ear veins of guinea-pigs were found to be very superficial, and there was a tendency to insert the needle too deeply. The needle, when correctly placed, could usually be felt to slide smoothly into the vein. The use of the magnifying spectacles, though not essential, was found to be an advantage. By using this method it was possible to give up to 30 daily injections each consisting of 5 ml. of fluid to the same guinea-pig.

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* Nuffield Dominions Demonstrator in Pathology.

EXPLANATION OF PLATE.

FIG. 1.—The apparatus used for the injection of the ear vein of a guinea-pig.

FIG. 2.—An injection in progress. One side of the ear is steadied by a finger of an assistant's left hand. The operator steadies the other side of the ear.

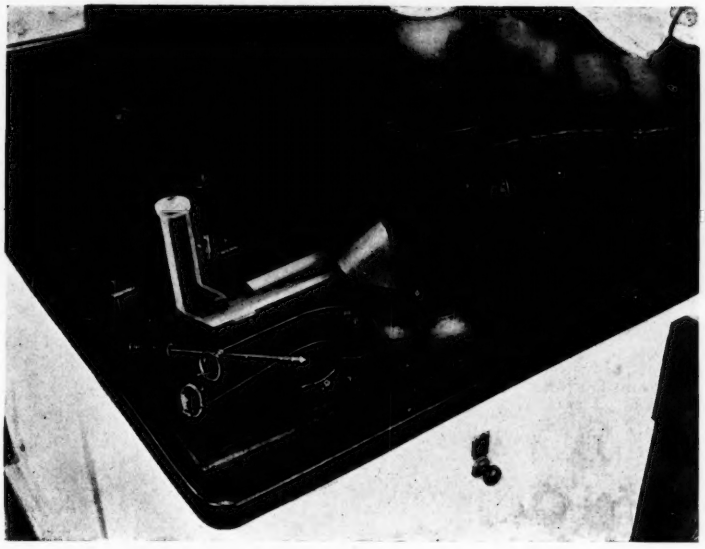

Fig. 1.

Fig. 2.

Markham and Kent.

ADSORPTIVE AND ENZYMIC BEHAVIOUR OF INFLUENZA VIRUS DURING THE O–D CHANGE.

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THE O and D phases of influenza virus were differentiated by Burnet and Bull (1943) on the basis of haemagglutinating properties and ability to multiply in different tissues of the chick embryo. As first isolated from the human infection, influenza A virus exists in the O (original) phase in which it agglutinates human, guinea-pig or pigeon red cells, but not fowl cells. During artificial cultivation it rapidly mutates to the D (derivative) phase, which agglutinates fowl cells to titres approximately equal to those found with other types of cell. Both phases grow in the amniotic cavity of the chick embryo, although D if present rapidly overgrows A. In the allantoic cavity D multiplies readily whereas O does not.

The behaviour during the O–D change varies somewhat in its finer points with the different strains of virus, and a number of preparations with properties intermediate between O and D have been described (Burnet and Stone, 1945; Anderson and Burnet, 1947; Dudgeon, Mellanby, Glover and Andrewes, 1948). By *in vitro* manipulations (Briody, 1948; Magill and Sugg, 1948), which reduce enzymic activity, these intermediate forms can be converted to virus with properties of the D phase but, as has already been pointed out (Burnet, Stone, Isaacs and Edney, 1949), virus with the full O character cannot be so modified *in vitro*. Such virus does not agglutinate fowl cells even at low temperatures, or under conditions where enzymic activity is excluded.

In view of the difference between O and D virus in ability to react with fowl cells and also with certain tissue cells, such as the allantoic cells of the chick embryo, it seemed possible that the two phases might be further differentiated by their behaviour with various soluble inhibitors for the virus haemagglutinin. Inhibitors are now available from a number of sources, and it has already been shown that they vary in their susceptibility to attack by different strains of virus (Stone, 1949*b*). There are two methods of studying the interaction between virus and inhibitor. The "inhibitor-sensitivity test" measures the inhibitory titre for virus which has been transformed to the indicator form (Stone, 1949*a*); in the test for "inhibitor-destroying activity" enzymic action of the respective virus is assessed against various inhibitors (Stone, 1949*b*). These tests have therefore been performed with preparations of O and D virus and a number of intermediate forms. Most of the work was carried out with the BEL strain which has been handled extensively in various stages of the O–D change.

MATERIALS AND METHODS.

Diluents.

Normal saline contained 0·9 per cent sodium chloride in distilled water.

Citrate-borate saline contained 10 per cent by volume of borate buffer (prepared

according to Palitzsch, see Clark, 1920) at pH 8·5 and 0·2 per cent sodium citrate in normal saline.

Calcium-acetate saline consisted of isotonic sodium acetate-acetic acid buffer, adjusted to pH 6·2 and 0·1 per cent calcium chloride.

Red cells.

Fowl and *pigeon* cells were from individual birds. Blood was taken from the wing vein into citrated saline. *Human* cells were from group O donors, the blood being defibrinated on collection. All cells were washed twice with normal saline and made up to 1 per cent suspension by volume in normal saline.

Inhibitors.

Chick embryo lung suspension was prepared from 14- to 16-day-old chick embryos. Lungs were removed with aseptic precautions, ground with alundum and suspended in normal saline (1 ml. per lung). The suspension was centrifuged lightly, the supernatant fluid removed and stored at 4°.

Ovomucin was prepared according to Gottschalk and Lind (1949). The titres refer to a stock solution containing 0·2 per cent (dry weight) in normal saline.

Human ovarian cyst mucoid was prepared by the method of McCrea (1948) The steps of the purification process were briefly as follows: (1) pseudo-mucinous cyst fluid, diluted with an equal volume of distilled water, was boiled to coagulate the bulk of inert material; (2) to the supernatant fluid were added 0·05 parts of sodium acetate and 1·5 parts of ethanol at room temperature; (3) the precipitate formed was taken up in distilled water and shaken with changes of chloroform to remove contaminating protein; (4) the inhibitor was re-precipitated with ethanol, and dried from the frozen state. The stock solution was of 1 per cent strength.

Virus.

The BEL strain of influenza A, isolated during a Victorian epidemic (Burnet, Beveridge, Bull and Clarke, 1942), was used throughout. *O-phase* virus was obtained by amniotic inoculation of material which had been maintained in the O phase during 26 amniotic passages at limiting dilution by the method described by Burnet and Stone (1945). *D-phase* virus was 44th allantoic passage from early O-phase material.

Inoculation of eggs.

Standard techniques described by Beveridge and Burnet (1946) were used throughout. Thirteen-day-old fertile hen's eggs were inoculated by the *amniotic route*. After incubation at 35° for 42 hours they were examined at 24-hour intervals by withdrawing a sample of amniotic fluid and testing for agglutination of human or pigeon cells. As soon as infection was detected the egg was opened and the amniotic fluid and lungs collected. Infected lung suspensions were prepared as described above for normal lungs. *Allantoic* inoculations were made in 11-day-old eggs. The eggs were incubated at 35° for 42 hours and samples of allantoic fluid tested for agglutination of human or pigeon red cells. Negative eggs were re-tested on the third and fourth days.

Haemagglutination titrations.

Doubling dilutions of virus were made in 0·25 ml. volumes of normal saline and an equal volume of 1 per cent red cells added to each tube. The pattern of cells was read after complete settling at room temperature (20–24°) or 4°, as specified in the text. Partial agglutination was taken as the end-point, and the titres expressed as the reciprocal of the end-point dilution.

Preparation of indicator virus.

Virus was diluted with four volumes of citrate-borate saline and heated at 56° for 30 minutes.

Titration of inhibitor-sensitivity of virus.

Doubling dilutions of inhibitor were prepared in 0·25 ml. of normal saline. An equal volume of a dilution of virus containing 5 agglutinating doses was added to each tube and, after holding the mixture for 30 minutes at room temperature, 0·25 ml. of a 1 per cent suspension of red cells. The test was read by the pattern of haemagglutination after a further hour at room temperature, partial agglutination being taken as the end-point.

Titration of inhibitor-destroying activity of virus.

Doubling dilutions of virus were prepared in 0·25 ml. volumes of calcium-acetate saline, and to each was added an equal volume of a standard dilution of inhibitor (containing 5–10 inhibitory doses, each dose being capable of inhibiting 5 ADs of the indicator virus used). After incubation for one hour at 37° the mixture was heated at 65° for 30 minutes to destroy the virus haemagglutinin, then cooled to room temperature. One drop of appropriate indicator virus containing 5 agglutinating doses was added to each tube, and after 30 minutes at room temperature 0·25 ml. of a 1 per cent suspension of fowl cells. The test was read by the pattern of haemagglutination after settling at room temperature (1 hour), partial agglutination being taken as the end-point.

EXPERIMENTAL.

Comparison of O and D Phases.

Inhibitor-sensitivity.

Virus in its indicator form was tested for inhibition by material of both fowl and human origin. A suspension of lungs from normal chick embryos was included with the inhibitors, as this tissue supports the growth of both O and D virus. Other inhibitors tested were ovomucin and human ovarian cyst mucoid.

TABLE I.—*Inhibitor-Sensitivity of O and D Viruses.*

Indicator virus.		Inhibitory titre.		
Phase.	F/P ratio.*	Chick lung.	Cyst mucoid.	Ovomucin.
O	12/400	<10	35,000	<100
D	640/560	640	40,000	6,000

* Ratio of haemagglutinin titres with fowl and pigeon cells at 4° given by virus before conversion to indicator. Inhibition tests were carried out with pigeon cells.

Since the preparation of indicator virus involved heating at 56°, infected chick embryo lung suspensions were used as the source of O virus in preference to amniotic fluid to avoid excessive precipitation. The D virus tested was an allantoic fluid preparation (44th allantoic passage). Fowl cells as used in the standard technique of inhibitor titration were obviously unsuitable for tests with O virus so that all experiments were made with human or pigeon cells.

It was found that cyst mucoid inhibited both O and D virus to approximately equal titres. However, although ovomucin or chick lung suspension inhibited D virus, they had little or no effect on virus in the O phase. It was shown in preliminary tests that the results were the same, using either human or pigeon cells and, owing to their more rapid settling, pigeon cells were adopted in further experiments. Table I gives the titres obtained with typical preparations of O and D virus.

Inhibitor-destroying activity.

This technique measures the dilution in which a sample of virus is capable of destroying a standard amount of inhibitor for the indicator virus used. The titre obtained for a given virus preparation depends on the position of the indicator virus in the inhibitor-gradient (Stone, 1949*b*).

A difficulty encountered in these experiments was the presence of inhibitor in some of the virus preparations which were being tested for enzymic activity. This was revealed only with certain indicators. It was essential therefore to test first a comparable series of virus dilutions against the indicator to be used, without adding the standard dose of test inhibitor. The samples were heated at 65° as in the standard test to destroy virus haemagglutinin before adding indicator virus and red cells.

TABLE II.—*Residual Inhibitor in Preparations of O Virus.*

Virus-containing fluid.		Inhibitory titre.*	
Source.	F/P ratio 4°.	Indicator—BEL D.	Indicator—BEL intermediate.
Amniotic fluid	15/320	<10	<10
	12/120	15	<10
	12/160	15	<10
Lung suspension	30/320	<10	<10
	<5/1280	100	<10
	<5/1280	120	<10
Control—normal lung suspension	—	480	200

* The virus was destroyed by heating for 30 minutes at 65° before the test.

Table II gives the titres of several preparations of O virus, both amniotic fluid and lung material, with indicators prepared from full D virus and an intermediate phase virus (F/P ratio at room temperature 280/640). It will be seen that the fluids varied in their inhibitory effect on the D indicator but were all inactive with the intermediate indicator. In cases where inhibition occurred it

was impracticable to test for inhibitor-destroying activity except in dilutions above the inhibitory titre. This complication did not arise with preparations of D virus, which were consistently negative against both indicators.

Table III shows the inhibitor-destroying activity of O and D virus for the inhibitors chick lung suspension, ovomucin and cyst mucoid. Both O and D virus destroyed the inhibitory activity of cyst mucoid for indicator-BEL D but, while D virus was active against the other inhibitors, O virus was inactive against embryo lung and ovomucin as tested with this indicator. O appeared to be as effective as D in destroying the inhibitor in embryo lung or ovomucin for indicator prepared from virus in the intermediate phase.

TABLE III.—*Inhibitor-destroying Activity of O and D Viruses.*

Virus.			Indicator-BEL D.			Indicator-BEL intermediate.	
Phase.	Source.	F/P ratio 4°	Chick lung.	Ovo-mucin.	Cyst mucoid.	Chick lung.	Ovo-mucin.
O	Amniotic fluid	15/320	<10	<10	480	200	160
O	Lung	30/320	<10	<10	320	160	120
D	Allantoic fluid	1000/1000	30	320	320	640	320

Although O virus did not destroy the inhibitor in chick lung for the indicator-BEL D in the standard test, lungs which had been infected with O virus frequently showed a considerable reduction in their inhibitor (*cf.* Table II). To test whether longer incubation would enable O virus to act on the lung inhibitor *in vitro*, normal chick lung suspension was incubated with an equal quantity of an O-infected lung suspension for 18 hours at 37°. The inhibitor for indicator-BEL D was reduced from a titre of 240 to 40, an effect comparable with that occurring *in vivo*.

Virus Preparations Intermediate between O and D.

The difference in the properties of O and D virus described in the preceding section was obtained using O virus prepared by amniotic passage and D virus which had received numerous passages in the allantoic cavity. In order to study the transition from the O to the D type of behaviour with inhibitors, a fresh series of passages adapting O to allantoic growth in the D phase was undertaken. To initiate the series a preparation of O virus was titrated by the amniotic and allantoic routes in parallel. The virus used was a suspension of chick embryo lung which had been absorbed with fowl cells according to the technique devised for obtaining satisfactory O seed virus (Burnet and Stone, 1945).

It will be seen from the results in Table IV that this material gave a proportion of amniotic infections in high dilutions, whereas only a few late positives at low dilutions were obtained in the allantoic cavity. F/P haemagglutinin ratios at room temperature and at 4° were determined on all infected fluids. Although no fluids in the full D phase were obtained in the amniotic titration, eggs inoculated with the lower dilutions or late positives in somewhat higher dilutions gave some agglutination with fowl cells (intermediate character). The positive allantoic

fluids must be classed as intermediate phase virus on haemagglutinating properties (Table V), but can be divided into two types. Three fluids gave F/P ratios equal to 1 at 4°, although they were lower at room temperature. These can be considered as close to the D phase. One fluid (egg 54687) showed a low F/P ratio (1/5) even at 4°, thus resembling O virus in its haemagglutinating properties. The fifth fluid was of very low titre.

TABLE IV.—*Comparative Amniotic and Allantoic Titration of O Virus.*

Dilution of inoculum.	Amniotic route.			Allantoic route.		
	2 days.	3 days.	4 days.	2 days.	3 days.	4 days.
10^{-7}	0/4	1/4	1/4	..	..	..
10^{-6}	0/5	1/5	1/5	0/4	0/4	0/4
10^{-5}	0/5	2/4	3/3	0/3	0/3	0/3
10^{-4}	1/4	2/4	3/3	0/3	0/3	0/3
10^{-3}	2/5	4/5	5/5	0/4	0/4	2/4
10^{-2}	5/5	..	..	0/4	3/4	3/4

Ratios are the number of eggs infected over the number surviving after incubation for the time shown. Infection was determined by agglutination of pigeon cells.

TABLE V.—*Haemagglutinin Titres of First Passage Allantoic Fluids.*

Dilution of inoculum.	Egg no.	F/P ratio.	
		At 4°.	At 20°.
10^{-3}	54684	80/80	15/35
10^{-3}	54685	120/200	20/60
10^{-2}	54687	<10/50	<10/80
10^{-2}	54688	120/120	30/80
10^{-2}	54689	<5/10	<5/10

These positive allantoic fluids were tested for their inhibitor-sensitivity, using the technique which differentiated O and D virus in the preceding section, viz., inhibition by chick embryo lung inhibitor in the presence of pigeon cells. It was found that fluids which resembled D in F/P ratio at 4° were inhibited to high titre (300), as occurred with full D virus tested earlier. Fluid 54687, on the other hand, was only inhibited to low titre (18); i.e., it resembled O virus in this property as well as in haemagglutination.

Allantoic Passage of Intermediate Fluids.

Fluids 54687 and 54688, representing the two types of intermediate phase virus just described, were then passaged serially in the allantoic cavity. Eggs were examined for infection on the second and third days by testing the allantoic fluid for agglutination of pigeon cells. The infected fluids were titrated with fowl and pigeon cells at 4° and room temperature, and tested for sensitivity to inhibition by chick lung suspension in the presence of pigeon cells. As the latter test provided a clear-cut differentiation of the fluids it will be convenient to label them by this criterion. Virus which was sensitive to inhibition will be referred

to as inhibitor-sensitive, while fluids which were not inhibited will be designated non-sensitive. At each passage the virus was subinoculated in a range of dilutions so that growth at the limit of infectivity could be studied as well as that from a heavy inoculum.

The results of passage of the inhibitor-sensitive fluid, 54688, will be reported first. It will be seen from Table VI that most infected fluids produced from this seed gave haemagglutinin ratios typical of D virus stock. Two fluids gave ratios at room temperature of 1/2 only. All fluids tested for inhibition by chick embryo lung inhibitor against pigeon cells were of the "inhibitor-sensitive" type. The positive fluid from the 10^{-6} group was used for the next passage in dilutions ranging from 10^{-2} to 10^{-8}. At all dilutions there were occasional fluids with haemagglutinin ratios ranging from 1/2 to 1/1 even at 4°. All fluids were inhibitor-sensitive.

TABLE VI.—*Second Allantoic Passage (Inhibitor-Sensitive Type) from Fluid* 54688 *of Table V.*

Dilution of inoculum.	Number of eggs infected.	F/P ratio. 4°.	F/P ratio. 20°.	Inhibitory titre against chick lung.
10^{-7}	0/4	—	—	—
10^{-6}	1/4	30/100	20/140	700
10^{-4}	3/3	120/100	70/60	400
		800/640	320/280	—
		80/80	20/40	600
10^{-2}	2/2	200/240	140/140	640
		640/320	320/240	120

Virus from this line of passage was also tested for inhibitor-destroying activity using both chick lung suspension and cyst mucoid as inhibitors. Representative fluids from the second and third passages were active against both inhibitors when BEL D or an intermediate BEL fluid were used as indicators; i.e., they behaved exactly like the D 44th allantoic passage virus, described earlier. Thirteen fluids from the third passage gave similar results. None of these fluids contained residual inhibitor for indicator BEL D virus, although normal allantoic fluid is inhibitory for this indicator.

The "non-sensitive" line of passage was established from fluid 54687. The results of the second passage (Table VII) show that the haemagglutinin ratios at 4° were all less than 1/2 and even lower at room temperature. All fluids tested for inhibitor sensitivity were found to be non-sensitive. Several could not be tested because of their low titre. The passage was continued for five further generations. Occasional fluids were obtained with haemagglutinin ratios at 4° between 1/2 and 1/1. However, subpassage of these again yielded a majority of fluids with a lower ratio. The non-sensitive character was maintained without exception over the six passages.

Inhibitor-destroying titrations on these fluids were unsatisfactory as many of them contained residual inhibitor for indicator-BEL virus. Tests with several fluids which were not inhibitory showed that they had little or no destructive action on the inhibitor in chick lung suspension for indicator-BEL D, but were active against cyst mucoid. The fluids were not inhibitory for the indicator

TABLE VII.—*Second Allantoic Passage (Inhibitor-Non-Sensitive Type) from Fluid* 54687 *of Table V.*

Dilution of inoculum.	Number of eggs infected.	F/P ratio. 4°.	F/P ratio. 20°.	Inhibitory titre against chick lung.
10^{-7}	0/3	—	—	—
10^{-6}	3/3	<10/15	<10/35	—
		<10/18	<10/40	—
		50/140	20/280	6
10^{-5}	2/3	40/120	12/280	8
		<10/20	<10/100	—
10^{-4}	2/3	<10/<10	<10/15	—
		50/120	25/200	10
10^{-3}	3/3	<10/20	<10/80	18
		12/25	15/60	10
		18/50	<10/60	16
10^{-2}	2/2	<10/15	<10/20	—
		50/200	40/400	18

form of an intermediate type allantoic fluid virus, and were found to destroy the inhibitor in chick lung suspension, ovomucin or cyst mucoid for this indicator.

The properties of the two types of virus distinguished in the first allantoic passage were therefore maintained on subpassage. In summary, the type which appeared most frequently was (*a*) inhibited by chick lung suspension in the presence of pigeon cells, (*b*) showed enzymic activity against both chick lung inhibitor and cyst mucoid, (*c*) gave somewhat variable F/P ratios, but mostly equal to unity at 4°. The second type of virus differed from the above in that (*a*) it was not inhibited by chick lung suspension in the presence of pigeon cells, (*b*) showed little or no enzymic activity on the chick lung inhibitor, (*c*) mostly gave F/P ratios less than 1/2 at 4°. Allantoic inoculation of BEL O virus has been repeated and the appearance of these two types confirmed.

DISCUSSION.

The O and D phases of influenza virus which were known to vary in their agglutination of red cells, have now been shown to behave differentially against soluble inhibitors. In addition to failing to react with fowl red cells, the O virus agglutinin was not inhibited by chick embryo lung suspension or ovomucin. D virus, on the other hand, was inhibited to high titre by these materials. Only human ovarian cyst mucoid was inhibitory for both phases of virus. Similar differences in enzymic activity on these inhibitors were demonstrable, the O virus having less action than the D on the fowl material, while both acted on human cyst mucoid. The bulk of the experiments was done on the BEL strain of influenza A (Burnet *et al.*, 1942), but the main points have been confirmed with a recently isolated A-prime strain, Ocean Island (Isaacs, Edney, Donnelley and Ingram, 1950).

When O virus was inoculated allantoically, most of the resulting fluids reacted with inhibitors like the fully adapted D phase. A few of those intermediate

between O and D in haemagglutinin reactions resembled O in failing to be inhibited by and having little enzymic action on chick embryo lung inhibitor or ovomucin. These properties were retained unchanged throughout six egg-passages. The virus therefore appears to be a stable mutant not previously recognized. Whether it occurs with other strains is not known. Anderson and Burnet (1947) have described a variant of IAN (influenza A) virus which was stable on allantoic passage and named at that time IAN-O, as it gave the O type of haemagglutination reactions when tested at room temperature. However, as this strain agglutinates fowl cells to full titre at 4° (Burnet *et al.*, 1949), it cannot be considered in the O phase. When tested for inhibitor-sensitivity it behaved like BEL-D virus.

Results of the two tests used in this work, inhibitor-sensitivity and inhibitor-destroying activity, indicate weaker action of O-phase virus on certain inhibitors, especially chick embryo lung suspension. Nevertheless, O virus does react with these inhibitors, as can be shown clearly by appropriate methods.

If suitable indicator virus—close to the O phase in character—is used for detecting residual inhibitor, it is found that O virus has considerable action on the chick lung inhibitor. This action is not revealed with D indicator-virus. These results emphasize again the existence of a gradient in the enzymic inactivation of soluble inhibitor, already observed in the comparison of various strains of virus (Stone, 1949*b*), and similar to that occurring in the agglutinability of red cells (Burnet, McCrea and Stone, 1946). In the present case the difference is between variants of the same strain, the gradation from weakly to strongly reacting virus corresponding to the steps from O through the intermediate phases to D. No evidence was obtained that O and D virus differed in position in the gradient for the human cyst mucoid inhibitor.

The test for inhibitor-sensitivity which differentiated sharply between O and D virus employs the three-component system: virus, chick embryo lung inhibitor, pigeon or human red cells. O virus combined preferentially with the red cell, D virus with the inhibitor. Tests with fowl cells could not be made for O virus, since agglutination characteristically fails to occur. However, certain intermediate-phase fluids were available which, although they resembled O virus in the standard inhibitor-sensitivity test with pigeon cells, gave some agglutination of fowl cells, especially after conversion to the indicator form by heating. When tested for inhibition by chick lung suspension in the presence of *fowl* red cells, these viruses were inhibited to high titre, showing that they combined with the inhibitor in preference to fowl cells. These findings are a further manifestation of the lower affinity of such viruses for fowl cells, as compared with pigeon cells, a phenomenon shown more directly in the haemagglutination titres obtained with the two types of red cell.

The present study extends the range of observations which characterize O–D behaviour. All differential properties, agglutination of fowl cells, growth in the allantoic cavity, greater reactivity with certain inhibitors from the chick embryo, are possessed by the D-phase virus and lacking in the O phase. Each of these features is related to the firmness of attachment to biological surfaces, be it red cell, infectible cell or soluble mucoids, and could be attributed to a specific configuration on the viral surface. On the available evidence a reasonable interpretation can be given for the fact that D-phase virus multiplies more readily than O in chick embryo tissues ; but there is no point of difference which could explain why the O phase is the human pathogen. However, this difference

might still lie in a specific affinity of O virus for the receptor on the human host-cell. Such a problem will not necessarily be solved by studying inhibitors in tissue extracts, since these would not contain only the cellular receptor with which the virus makes its initial contact during natural infection. It is telling in this regard that chick embryo lung inhibitor with which O virus reacts poorly is prepared from a tissue which supports the growth of virus readily.

SUMMARY.

Mutants arising during the O–D change of an influenza A virus strain have been tested for their reactions with three types of haemagglutinin inhibitor.

O-phase virus is enzymically active against human ovarian cyst mucoid, but not against ovomucin or ground chick embryo lungs. D-phase virus acts upon all three inhibitors.

After transformation to the indicator form by heating, O virus is inhibited only by the human mucoid, while D virus is inhibited both by human and avian mucoids.

During allantoic adaptation O virus yielded two types of mutants besides the full D phase. One, which was close to D in its haemagglutinin reactions, resembled the D phase by being active on both human and avian mucoids. On passage this virus regularly yielded the D phase.

The second type, agglutinating fowl cells less readily, resembled the O phase by acting only on human mucoid. This mutant, which grew readily in the allantoic cavity, retained its properties during six consecutive passages.

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SOME HISTOLOGICAL ASPECTS OF FORMALIN "ARTHRITIS" IN RATS.

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SELYE (1949) described a method of inducing experimental "arthritis" in rats by the injection of formaldehyde beneath the plantar aponeurosis of the hind paws. This method has been used by other authors to estimate the anti-arthritic effect of various substances. The anti-arthritic effect has been "measured" by the ability of the test substance to reduce the oedema which develops rapidly after injection of formalin. However, since oedema is only one of the manifestations of inflammation, and since very little information has been published concerning the histological changes induced in the joint and its neighbouring tissues by injection of formalin into the plantar pad, it was considered worth while making a histological examination of the effects of this procedure, not only in the injected limbs but also in the contralateral limbs. At the same time changes in the cortical lipoid of the adrenal glands were noted.

METHOD.

Sixteen rats were injected in the plantar pad of the left foot with 0·1 ml. of a 2 per cent solution of formaldehyde, and then pairs of rats were killed, and their tissues fixed in formaldehyde, at the following periods of time: 5 minutes, 1 hour, 3 hours, 8 hours, 24 hours, 3 days, 1 week, 2 weeks.

The left and right ankle-joints were fixed for 48 hours, then washed in tap water and decalcified electrolytically. Paraffin sections were stained with haematoxylin and eosin and in some cases with azan and aniline blue. The adrenals were washed for 5 hours; frozen sections were cut and stained with Sudan black to demonstrate fat and lipoid.

In addition the ankle-joints and adrenals of four rats which had been injected with formaldehyde one month previously were examined. The left ankle-joints of nine other rats injected a month previously were also examined, and so were those of three rats which had had three injections of formaldehyde into the left hind plantar pad at two-day intervals and had been killed a month following the last injection.

RESULTS.

The foot began to swell during the first hour after injection of the formalin, and this continued until eight hours and then began to decrease. This decrease continued, the ankle-joints of most animals returning to normal somewhere

between 1 and 3 weeks after the injection. Some swelling was present in the joints of some animals over a month after injection.

In the ankle-joints of animals killed five minutes after the formalin injection little change could be seen, but there seemed to be a swelling of parts of the synovial membrane in one animal.

At the end of an hour very little histological change was yet visible. There was some microscopical indication however of the oedema, which was by then visible macroscopically. Swelling of cells of the synovial membrane could be seen in the left ankle-joint of one animal; in the other a granular precipitate in some of the synovial cavities suggested an increase in the solid matter of the synovial fluid (? protein), which presumably indicated some change in the permeability of the synovial membrane at this stage.

No significant changes could be seen in the right ankle-joint of either animal.

After 3 hours there was considerable increase in the granular precipitate (which stained basophilically) in the synovial cavities of the left ankle-joints of both animals. This was also found in the right ankle-joints.

Increase in the fragility of the capillary walls was also evidenced by the presence of free red cells in the dermal fascia near the site of the injection of the formalin. The first cellular evidence of inflammation occurred in one animal in the form of a small infiltration of macrophages into the dermal fascia and into some of the peri-articular tissues. Although the majority of these cells were macrophages many neutrophils were also present.

EXPLANATION OF PLATE.

FIG. 1.—Lipoid preparation (Sudan black) of adrenal of rat injected with formalin in the plantar pad of the left hind foot 5 minutes previously. Distribution of lipoid normal.

FIG. 2.—Lipoid preparation of adrenal of rat injected with formalin in the plantar pad of the left hind foot 3 hours previously. Considerable loss of lipoid from zona glomerulosa. Lipoid distribution of rest of cortex unaffected.

FIG. 3.—Lipoid preparation of adrenal of rat injected with formalin in the plantar pad of the left hind foot 8 hours previously. Considerable loss of lipoid from zona reticularis as well as from zona glomerulosa.

FIG. 4.—Lipoid preparation of adrenal of rat injected with formalin in the plantar pad of the left hind foot 24 hours previously. Considerable loss of lipoid from all zones of adrenal.

FIG. 5.—Lipoid preparation of adrenal of rat injected with formalin in the plantar pad of the left hind foot 1 week previously. Lipoid of glomerulosa and fasciculata zones back to normal. Reticularis still shows slight loss of lipoid. Differences in width of cortex are not significant.

FIG. 6.—Skin and surrounding tissue of left hind paw of rat in which formalin has been injected 3 days previously. Destruction of epithelium and extensive cellular infiltration of the skin and dermal fascia is apparent.

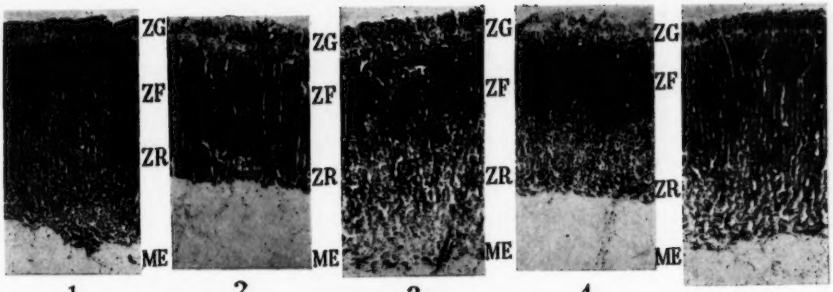
FIG. 7.—Showing infiltrating cells in muscle near ankle-joint. Animal has been injected 1 week previously with formalin. The majority of these cells are round cells.

FIG. 8.—Section through skin and ankle-joint of rat near site of injection of formalin 1 week previously. Infiltration of cells between muscle fibres can be seen. This infiltration passes down to but does not pass through the joint ligament seen near the bottom of the section.

FIG. 9.—Showing well-developed peri-articular fibrous tissue in ankle-joint of rat injected 4 weeks previously with formalin.

FIG. 10.—Showing types of invading cells in sub-dermal fascia near ankle-joint of rat injected with formalin 1 week previously. Round cells and polymorphs may be seen, also the beginnings of fibre production by fibroblasts.

ZG. Zona glomerulosa. ZF. Zone fasciculata. ZR. Zona reticularis. ME. Medulla. E. Epithelium of skin. F. Regions of fibroblastic activity (multiplication and fibre production). M. Muscle. DF. Dermal fascia. I. Areas of cellular infiltration. L. Joint ligament.



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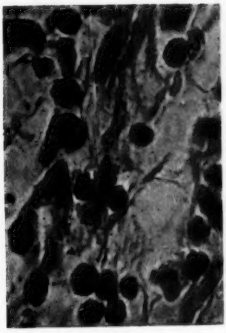

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At 8 hours, when the oedema of the ankles was at its height, the synovial fluid of the left ankle-joints of both animals showed considerable increase of solid matter. In one animal macrophages were also present in the synovial cavities and in the other some fibrin could be seen. The cells of the synovial membranes were swollen in some regions. Cellular infiltration was apparent in the peri-articular tissues of the left ankle-joints of both animals at this stage but was not yet very extensive. It was confined to the dermal fascia near the site of injection in one animal, and in the other it had spread to the joint capsule and was beginning to travel along the capsule parallel with the fibres. There was a slight fibroblastic proliferation in the dermal fascia of one animal.

The right ankle-joints of both animals at 8 hours showed some slight cellular infiltration, but the synovial cavities appeared normal.

At 24 hours there was still fibrin and increased granular deposits in the synovial fluid, and considerable increase in the infiltration of the peri-articular tissues together with a slight fibroblastic proliferation.

After 3 days the increase of solid matter in the synovial fluid was still obvious, and granular deposits were also found at the site of extra-articular accumulations of fluid.

In one animal parts of the synovial membranes had become infiltrated and fibroblastic proliferation in the dermal fascia had increased.

The right ankle-joints of the animals showed little change from normal.

By one week there was considerable cellular infiltration of the dermal fascia extending some distance from the site of the injection. At this stage too there was fibroblastic proliferation in the same site. There was also some infiltration of, and fibroblastic proliferation in, the peri-articular tissues. The synovial fluid and membranes appeared to have returned to normal.

The right ankle of one animal showed a patch of fibroblastic proliferation in the dermal fascia, while that of the other animal appeared normal.

A fortnight after injection the left ankle-joint of one animal showed extensive cellular infiltration of the dermal fascia, which, however, did not extend through to the joint. There was very little fibroblastic proliferation or activity. In the other animal the left ankle-joint showed considerable infiltration and fibroblastic activity in the same region.

In neither case had these inflammatory processes forced their way through the peri-articular tissues into the joint cavity itself. The synovial fluid and membranes and the articular surfaces in right and left ankle-joints of both animals appeared quite normal.

Of the 13 animals killed one month after injection of the formaldehyde, seven seemed quite normal and all signs of cellular infiltration and fibroblastic reaction had disappeared. Four still showed some signs of both these processes, and two had left ankle-joints which were still heavily infiltrated and in which fibroblastic activity continued.

Of three rats which had received three injections of formaldehyde into the same foot and were killed one month after the last injection, two showed more or less normal ankle-joints and the third still showed considerable cellular infiltration of, and considerable fibroblastic activity in, the dermal fascial and the peri-articular tissues. There was also some infiltration of the synovium, and granular deposits (from the synovial fluid) were present in the joint cavities. The articular cartilage was unaffected.

The Adrenals.

The changes in the adrenals can be summarized as follows :

5 minutes	2 animals	Cortex has full load of lipoids in all zones.
1 hour	,,	Slight loss of lipoid from zona glomerulosa.
3 hours	,,	50 per cent loss of lipoid from zona glomerulosa and slight loss from zona reticularis.
8 ,,	,,	Considerable reduction of lipoid in all zones.
24 ,,	,,	Lipoids considerably depleted in all zones.
3 days	,,	Ditto.
1 week	,,	1 animal shows lipoids in all zones nearly normal. 1 animal shows zona glomerulosa normal, but reticularis and fasciculata zones not back to normal.
2 weeks	,,	All zones appear normal.
1 month	4 animals	2 animals have all zones with full load of lipoid. 2 animals show considerable depletion of lipoids particularly in zona glomerulosa and zona reticularis.

The changes in the last group can not be correlated with changes in the joints.

DISCUSSION.

It is noteworthy that apart from the initial changes in permeability of the synovial membrane as evidenced by the increased solid matter in the synovial fluid the inflammatory processes do not extend into the joint itself. Only in one or two animals was there even cellular infiltration into the synovial membranes. In fact the condition produced by formalin injection is not an arthritis but a peri-articular inflammation. It is obvious that treatment which rapidly reduces the oedema associated with such a procedure may not necessarily affect the processes of cellular infiltration and fibroblastic proliferation which follow it.

Selye found that several formaldehyde injections at intervals of a few days produced a " self-maintaining chronic inflammatary process," which continued to progress after formalin injections had been discontinued. Joints affected in this way were said by Selye to resemble the rheumatoid type of arthritis, and the acute oedematous joints to resemble the rheumatic type. Coutu, Selye and Gareau (1951) have described the histological changes which follow the injection of mustard powder into the plantar pads of rats. The peri-articular changes they describe resemble those described in the present paper but in addition they found that granulation tissue penetrated into the joint cavity and became progressively organized. They also found degeneration of the cells and erosion of the articular cartilage. Nothing of this sort was found in the present work.

The difference in the two results may be explained by the more prolonged irritation produced by the mustard powder.

The right ankles show changes in the synovial fluid (increased solid matter) at an early stage, but this in most cases passes off rapidly. Right ankles of some animals have been examined from time to time, however, a month after injection and found to show appreciable cellular infiltration around the joint.

The changes in the adrenal lipoids show that there is no effect from the formalin injections in the first five minutes, but that discharge of these lipoids is beginning by the first hour. It is of interest that the first signs of loss of lipoid show in the zona glomerulosa, a zone in which, Deane and Greep (1946) have brought forward evidence to show, the salt and water controlling hormones (desoxycorticosterone or related substances) are produced. Loss of lipoid is next shown by the zona reticularis, and there is considerable loss from both these zones before there is any obvious loss of lipoid from the zona fasciculata, where, according to Deane and Greep, the carbohydrate hormones are localized. There may be some significance in the fact that extensive discharge of lipoid from all zones which has occurred by 8 hours coincides with the beginning of cellular infiltration and fibroblast proliferation round the ankle-joints. The adrenals have regained most of their lipoid by the end of one week and appear quite normal again after a fortnight.

SUMMARY.

Injection of formaldehyde into the plantar pads of the hind feet of rats causes a peri-articular inflammation characterized by oedema of the peri-articular tissues, increase of solid matter in the synovial fluid, cellular infiltration of the dermal fascia and the peri-articular tissues and increased fibroblastic activity. By one month most of the joints have returned to normal, but some still show considerable cellular infiltration and fibroblastic activity. There appears to be some correlation between lipoid changes in the adrenal and the onset of cellular inflammatory changes in the joints.

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IMMUNOLOGICAL STUDIES ON A HIGHLY PURIFIED CHOLINESTERASE.*

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PREVIOUS reports from this laboratory indicate that there is a close correlation between erythrocyte permeability and the activity of the acetyl choline-cholinesterase system (Greig and Holland, 1949; Holland and Greig, 1950). Inhibition of erythrocyte cholinesterase activity by an inhibitor such as physostigmine produces marked changes in the permeability of erythrocytes to Na, K, water and haemoglobin. When tested with a large number of enzymes physostigmine was shown to inhibit only cholinesterase in small concentrations; however, the possibility exists that this drug could also inhibit other enzyme systems.

Since this possibility exists, we thought that by use of immunological methods we might be able to prepare a specific inhibitor of cholinesterase by immunizing animals with the purest preparation of erythrocyte cholinesterase available. If the protein cholinesterase is antigenic and the antibodies so produced inhibit the activity of the enzyme, then we would expect this specific anticholinesterase to produce changes in permeability of the species of erythrocytes which were used as the source of the enzyme. This report represents the results of this study.

METHODS.

Ox erythrocyte cholinesterase was obtained from Winthrop-Stearns, Inc.† The enzyme is prepared from ox erythrocyte stroma by incubating the material in dilute NaOH (pH 8–9) solution for 24–36 hours at 37·5°. The enzyme is further purified by repeated fractional $(NH_4)_2SO_4$ precipitations. This preparation is water-soluble and contains human serum as a preservative. 1 mg. of the material corresponds to 1000 units, a unit being defined as that quantity of enzyme which will liberate 1 c.mm. carbon dioxide per minute from a Ringer-bicarbonate buffer equilibrated against 5 per cent carbon dioxide in nitrogen at pH 7·4 and at 37·5°, when the initial substrate (acetyl choline) concentration is 0·0092 M.

Seven large male rabbits were immunized with this preparation. A total of twelve intravenous injections were given at two-day intervals, the total dose being between 30–50 mg. The rabbits were exsanguinated at varying intervals after the initial injection and the sera pooled and kept in a frozen state without a preservative.

Cholinesterase activity was determined manometrically, using the Warburg technique. Determinations were carried out at 37° in a Ringer's bicarbonate medium. The final volume was 2 ml. and acetyl choline bromide in a final concentration of 10^{-2} M was employed as substrate. Quantities of packed ox erythrocytes, antiserum and guinea-pig serum used are given in the Table.

The results of only a few representative experiments are presented.

* Funds for carrying on this work were supplemented by grants from the Mallinckrodt Chemical Works and the Upjohn Company.

† Prepared according to U.S. Pat. 2,475,792.

TABLE.—*The Effect of Antiserum and Fresh Guinea-pig Serum on the Cholinesterase Activity and Permeability of Ox Erythrocytes.*

Exp. No.	Duration of experiment (min.).	Vol. of serum added (ml.).	CO_2 evolved. (c.mm.) Normal serum.	CO_2 evolved. (c.mm.) Immune serum.	Per cent Inhibition.	Titre of antisera.	Vol. of g.p. serum added (ml.).	Enzyme preparation (ml.).	Haemolysis.
1	100	·1 (fresh)	69	69	0	1–500	...	·02 of packed cells	0
		·2 (fresh)	70	59	14				+
2	40	·1 ,,	52	40	23	1–1500	...	Ditto	++
		·2 ,,	52	21	60				+++
3	40	·2 (heated)	41	41	0	1–1500	...	,,	0
4	70	·2 ,,	97	97	0	1–1200	...	,,	0
5	40	·2 (5 days old)	65	50	23	1–3000	...	,,	+
6	60	·3 ,,	44	24	45	1–3000	...	,,	++
7	45	·2 (heated)	44	44	0	1–2500	·1 (undiluted)*	,,	0
8	45	·2 ,,	79	77	0	1–2500	·1 ,, *	,,	0
9	50	·2 ,,	34	28	18	1–2500	·1 ,, †	,,	Complete haemolysis
		·3 ,,	36	25	31				
10	50	·2 ,,	40	36	10	1–2500	·1 ,, †	,,	Complete haemolysis
		·3 ,,	60	25	58				

* Fresh guinea-pig serum in side bulb during equilibration.
† Fresh guinea-pig serum added to reaction flask during equilibration.

RESULTS.

In preliminary experiments it was noted that ox red cells in contact with the antiserum obtained from rabbits immunized with the purified ox erythrocyte enzyme preparation were much more fragile than those cells in contact with normal serum.

These findings suggested that the antiserum contained a haemolysin, and in subsequent experiments which are reported in this communication the haemolysin titre was followed by use of the complement haemolysis test. Haemolysin titres are expressed as the highest dilution of antiserum that gives perceptible haemolysis when an excess of complement is used. The haemolysin titre of each rabbit serum as well as of the pooled sera was determined. In experiments reported here only pooled antisera were employed. The haemolysin titre rose from 1/50 on the day previous to immunization, to 1/750 on the twelfth day of immunization and reached a maximum of 1/1250 to 1/3500 on the tenth day after the last injection. In a period of twenty-one days, the titre had fallen to 1/300 to 1/500. No agglutination of the ox red cells was observed except in dilutions of the antiserum less than 1/50.

The effects of antiserum on ox erythrocyte cholinesterase are given in the Table. Homologous antiserum caused 30–60 per cent inhibition of cholinesterase activity of ox red cells only under certain experimental conditions. Inhibition occurred when fresh unheated antiserum was used. Heated (56° for 30 minutes) antiserum had no effect. Inhibition occurred with heated antiserum when fresh guinea-pig serum was added to the ox cells prior to determination of enzyme activity. Heated guinea-pig serum had no effect. Guinea-pig serum alone produced no significant inhibition of cholinesterase activity. The antiserum in the presence of fresh guinea-pig serum had no effect on the cholinesterase activity of dog, cat or guinea-pig erythrocytes.

There was a close correlation between haemolysis in the experimental flask and inhibition of enzyme activity. For example (Exp. 7, 8), if acetyl choline, antiserum and ox red cells were added to the Warburg flask and the guinea-pig serum to the side bulb, and equilibrated at 37° for 30 minutes before the guinea-pig serum was added, no haemolysis of the red cells or inhibition of cholinesterase activity occurred. If (Exp. 9, 10), however, the fresh guinea-pig serum was added initially to the test system, both haemolysis of the red cells and inhibition of the enzyme activity occurred.

Both the inhibitor and haemolysin could be removed from the antiserum by ox erythrocytes, but not with human serum or human, dog, cat or guinea-pig red cells. Due to the extreme instability of the enzyme preparation in solution we were unable to obtain any consistent results when attempts were made to remove the inhibitor or haemolysin from the antiserum with the purified preparation.

We have investigated the effects of antiserum and fresh guinea-pig serum on the enzyme activity of the purified cholinesterase preparation. Inhibition of the cholinesterase activity occurred under the same experimental conditions as were employed in the case of intact ox erythrocytes, but the degree of inhibition was much less (25–30 per cent). In these experiments minute amounts of enzyme (·0025–·0050 mg. of protein in a final volume of 2 ml.) were used, and it may be that the antigen-antibody complex was more dissociated than was the case when intact ox erythrocytes were employed.

DISCUSSION.

In this communication we have reported the results of experiments attempting to produce a specific inhibitor of cholinesterase by immunizing rabbits with the purest cholinesterase available. Antiserum was obtained which inhibited the activity of this enzyme. Fresh antiserum produced 40–60 per cent inhibition but if the antiserum was heated or allowed to stand at 5° for several days the inhibition decreased considerably. The inhibitory power of the heated antiserum could be restored by addition of fresh guinea-pig serum (Exp. 7, 8, 9 and 10). Heated guinea-pig serum had no effect. We also observed that fresh antiserum would cause haemolysis of ox erythrocytes. This haemolytic property of the fresh antiserum could be destroyed by heating at 56° for 30 minutes, and was markedly reduced at 37° for one half hour. Under our experimental conditions there was a close correlation between haemolysis of the ox erythrocytes and inhibition of cholinesterase activity. The greater the degree of cholinesterase inhibition, the greater was the haemolysis observed. When physostigmine was employed as an inhibitor of cholinesterase activity similar effects were observed. We found a close correlation between cholinesterase inhibition and haemolysis of erythrocytes. Physostigmine in concentrations sufficient to produce 40–60 per cent inhibition of cholinesterase activity caused also complete haemolysis of red cells, while smaller concentrations of the drug produced decreasing amounts of haemolysis. Although we have not proved that the haemolysin and the anticholinesterase are the same, their activities closely parallel each other.

Cholinesterase is said to be situated in the cell surface (Paleus, 1947 ; Brauer and Root, 1945 ; Augustinsson, 1948) where many immunological phenomena are believed to occur. This assumption is based on the belief that the erythrocyte stroma represents the cell membrane. In case of the giant axon of the squid, cholinesterase has been shown to be located in or very near the surface of the cell (Nachmansohn, 1945).

Bordet in 1898 made the classical discovery that serum of rabbits immunized with foreign erythrocytes would cause agglutination and haemolysis of the homologous cells. He also pointed out that this haemolysis observed was dependent upon the presence of complement.

Fisher and Keogh (1950) presented data which suggest that the interaction of any antigen on the surface of a red cell with the corresponding antibody followed by complement will produce haemolysis. The observations of these authors have been extended by Adler (1950). Attempts were made by this author to reverse the process, by first sensitizing the erythrocyte with the antibody, and to induce lysis by adding such cell suspensions to serial dilutions of the corresponding antigen in presence of complement. Under these conditions, neither appreciable complement-fixation nor lysis occurred. Certain viruses are known to cause agglutination of red cells by combining with certain receptors on the surface of the cell (Hirst haemagglutination reaction). It has been known for some time that tannic acid has an effect on erythrocytes which is analogous to the action of amboceptor ; small concentrations of this substance produce agglutination of the cells but no haemolysis, while the addition of complement results in haemolysis (Landsteiner, 1946). These substances discussed above (antigens, viruses and tannic acid) apparently are combining with certain receptor groups on the cell surface. Whether cholinesterase is one of these receptor groups remains to be seen. Only in the case of the viruses is there any experi-

mental evidence which suggests that cholinesterase may play some role in the combination of these viruses with the receptor groups on the surface of the erythrocyte. Svec and Forster (1947) showed that extracts of several tissues (normal sera and human red cells) containing cholinesterases would prevent the agglutination of chicken red cells by influenza virus.

Numerous attempts have been made to isolate from red cells the antigen or antigens responsible for the production of haemolysins. The active material is present in red cell stroma, and under certain conditions can be separated from stroma, in a partially purified form (Delves, 1939). Balls and Korns (1918), by treating sheep erythrocyte stroma with dilute alkali, obtained a protein preparation which stimulated the production of haemolysins in very high titres. This procedure as described above would also elute cholinesterase. These authors thus may have been dealing with a cholinesterase preparation as their antigen. To our knowledge no one has tested these partially purified preparations of red cell stroma for enzyme activity. The preparation we have used in our experiments is a purified fraction of ox erythrocyte stroma that exhibits a very high cholinesterase activity. Could it be that cholinesterase is one of the antigens present in ox erythrocyte stroma which is responsible for the stimulation of the production of haemolysins in the rabbits immunized with the preparation containing the enzyme? Experiments reported in this paper suggest that this may well be the case.

SUMMARY.

The immunological properties of a purified preparation of ox erythrocyte stroma having a high cholinesterase activity are presented.

Antiserum was prepared which inhibited cholinesterase 40–60 per cent and caused haemolysis of ox erythrocytes. These effects were observed with fresh antiserum. The activity was lost on standing for 4–7 days at 5° or on heating to 56° for 30 minutes, and could be restored by the addition of fresh guinea-pig serum.

Evidence is presented which suggests that in the case of ox erythrocytes cholinesterase may be one of the antigens present in red cell stroma which stimulates the production of haemolysins when rabbits are immunized with stroma or fractions thereof.

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